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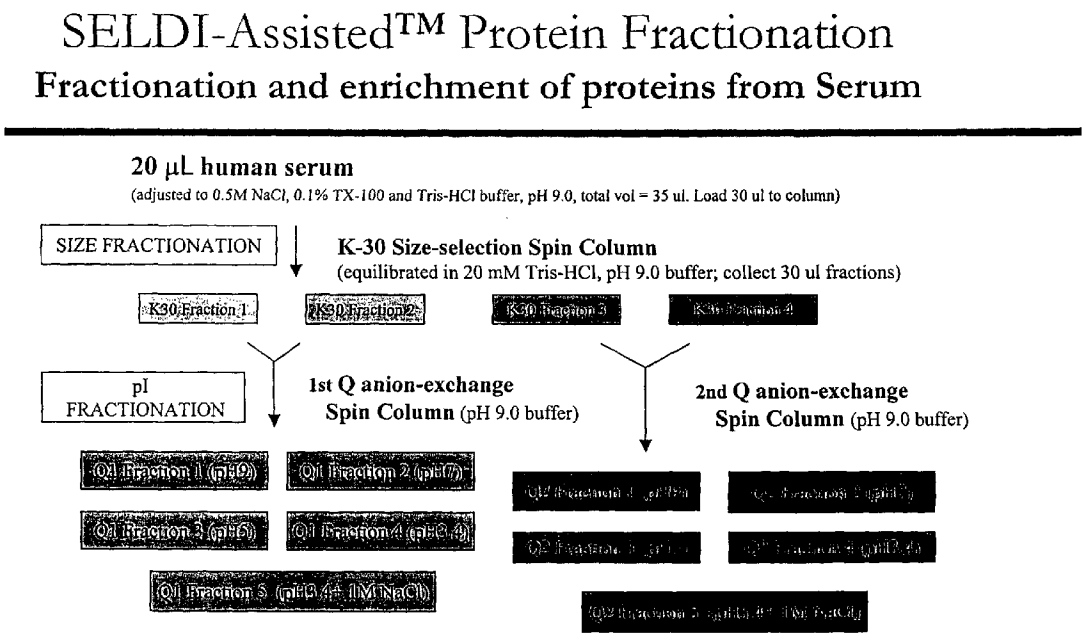

(Each Q column fraction is 150 ul)

(57) Abstract: The invention provides markers, methods and kits that can be used as an aid for breast cancer diagnosis using markers that are differentially present in the samples of breast cancer patients and a control (e.g., women in whom breast cancer is undetectable).

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HUMAN BREAST CANCER BIOMARKERS

CROSS-REFERENCES TO RELATED APPLICATIONS

Not applicable.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

Not applicable.

BACKGROUND OF THE INVENTION

Breast cancer is the most common fatal malignancy in women. About 15% of all women will be diagnosed with breast cancer during their lifetime. In the U.S., breast cancer is the third leading cause of death in women. Despite recent progress in early detection, as well as improved treatment, the mortality rate remains unchanged. Early diagnosis is the key to surviving breast cancer.

Typically, the detection of breast cancer involves an exam by a physician, a mammogram, and either a needle aspiration or biopsy. When the breast tissue forming the lump is removed, the tissue is examined for possible cancer cells.

A few protein markers have been used to aid the diagnosis and/or prognosis of breast cancer. For example, CA 15-3, a glycoprotein found in the breast, has been used as a marker to monitor recurrence of breast cancer and responses to various treatments. However, the CA 15-3 glycoprotein level is only informative for evaluating patients' condition before and 30 days after the surgical resection of the tumor. Other than in these few cases, this marker is not informative. For example, a third of breast cancer patients with metastasis have CA 15-3 concentrations within the normal range. Even after radical resection of the tumor, CA 15-3 exhibits a substantial variation at abnormal concentrations.

Another marker that has been used to diagnose breast cancer are the neu oncogenes (also known as HER-2 and c-erb-2). This gene is overexpressed in the tumor cells of about 40% women with breast cancer. The neu oncogene products can be elevated as much as 30 to 45-fold above normal levels in human breast tumor. However, there are problems associated with using this protein marker as a diagnostic tool for breast cancer. For example, false positives are often observed using this marker. Moreover, in

about 50% of patients, the plasma HER-2 levels do not show any response to treatment. Accordingly, the detection of these individual markers alone cannot be used as a diagnostic test as to whether the subject has a breast cancer.

The effectiveness of any diagnostic test depends on its specificity and selectivity. That is, what is the relative ratio of true positive diagnoses, true negative diagnoses, false positive diagnoses and false negative diagnoses? Methods of increasing the percent of true positive and true negative diagnoses for any condition are desirable medical goals. In the case of breast cancer, the present diagnostic tests are not completely satisfactory, in that they provide significant numbers of false positive and false negative results as described above.

There is some consensus in the medical community that better diagnosis will result from the discovery of more disease markers. Moreover, in an economy-conscious environment in which cost-effective medicine is an overriding concern, physicians treating cancer patients need convenient, efficient methods to rapidly diagnose breast cancer and to evaluate responses to therapy. The present invention meets this and other needs.

SUMMARY OF THE INVENTION

The present invention provides, for the first time, novel protein markers that are differentially present in the samples of breast cancer patients and in the samples of control subjects. The present invention also provides sensitive and quick methods and kits that can be used as an aid for diagnosis of breast cancer by detecting these novel markers. The measurement of these markers, alone or in combination, in patient samples provides information that diagnotician can correlate with a probable diagnosis of breast cancer or a negative diagnosis (*e.g.*, normal or disease-free). All the markers are characterized by molecular weight. The markers can be resolved from other proteins in a sample by, *e.g.*, chromatographic separation coupled with mass spectrometry, or by traditional immunoassays. In preferred embodiments, the method of resolution involves Surface-Enhanced Laser Desorption/Ionization ("SELDI") mass spectrometry, in which the surface of the mass spectrometry probe comprises adsorbents that bind the markers.

A first set of markers is identified from blood serum, and are capable of binding to a hydrophilic adsorbent (*e.g.*, silicon oxide) and other adsorbents. These markers include Marker BrH1: 6850 ± 14 Da; Marker BrH2: 8565 ± 17 Da; Marker

BrH3: 8920 ± 18 Da; Marker BrH4: 11180 ± 22 Da; Marker BrH5: 15220 ± 30 Da; Marker BrH6: 106080 ± 530 Da; and Marker BrH7: 117600 ± 590 Da. A second set of markers is also identified from blood serum. These markers are capable of binding to a metal chelate adsorbent (*e.g.*, a copper chelate adsorbent) and other adsorbents. These markers include Marker BrM1: 2804 ± 6 Da; Marker BrM2: 3390 ± 7 Da; Marker BrM3: 5890 ± 12 Da; Marker BrM4: 11900 ± 24 Da; and Marker BrM5: 51000 ± 260 Da. These markers are more frequently detected and/or are detected at an elevated level in breast cancer patients' samples than in the samples of control subjects (*e.g.*, women with a negative diagnosis).

While these markers were first identified from the blood serum sample, the sample from which they can be detected is not limited to a blood serum sample. These markers may be detectable in other types of samples, such as nipple aspirate, urine, tears, saliva, *etc.* Moreover, although the first and second set of markers were discovered using a hydrophilic adsorbent and a metal chelate adsorbent, respectively, the markers are capable of binding other types of adsorbents as described below. Accordingly, embodiments of the invention are not limited to the use of hydrophilic adsorbents and metal chelate adsorbents.

While the absolute identity of these markers is not yet known, such knowledge is not necessary to measure them in a patient sample, because they are sufficiently characterized by, *e.g.*, mass and by affinity characteristics. It is noted that molecular weight and binding properties are characteristic properties of these markers and not limitations on means of detection or isolation. Furthermore, using the methods described herein or other methods known in the art, the absolute identity of the markers can be determined.

Accordingly, in one aspect the invention provides methods for aiding a breast cancer diagnosis, the method comprising: (a) detecting at least one protein marker in a sample, wherein the protein marker is selected from Marker BrH1: 6850 ± 14 Da; Marker BrH2: 8565 ± 17 Da; Marker BrH3: 8920 ± 18 Da; Marker BrH4: 11180 ± 22 Da; Marker BrH5: 15220 ± 30 Da; Marker BrH6: 106080 ± 530 Da; Marker BrH7: 117600 ± 590 Da; Marker BrM1: 2804 ± 6 Da; Marker BrM2: 3390 ± 7 Da; Marker BrM3: 5890 ± 12 Da; Marker BrM4: 11900 ± 24 Da; and Marker BrM5: 51000 ± 260 Da; and (b) correlating the detection of the marker or markers with a probable diagnosis of breast cancer.

In one embodiment, the correlation takes into account the amount of the marker or markers in the sample and/or the frequency of detection of the same marker or markers in a control.

In another embodiment, gas phase ion spectrometry is used for detecting the marker or markers. For example, laser desorption/ionization mass spectrometry can be used.

In another embodiment, laser desorption/ionization mass spectrometry used to detect markers comprises: (a) providing a substrate comprising an adsorbent attached thereto; (b) contacting the sample with the adsorbent; and (c) desorbing and ionizing the marker or markers from the substrate and detecting the desorbed/ionized marker or markers with the mass spectrometer. Any suitable adsorbents can be used to bind one or more markers. For example, the adsorbent on the substrate can be a hydrophilic adsorbent (*e.g.*, silicon oxide), a metal chelating adsorbent, a lectin adsorbent.

In another embodiment, an immunoassay can be used for detecting the marker or markers.

In another embodiment, methods further comprise (a) generating data on the sample with the mass spectrometer indicating intensity of signal for mass/charge ratios; (b) transforming the data into computer-readable form; and (c) operating a computer to execute an algorithm, wherein the algorithm determines closeness-of-fit between the computer-readable data and data indicating a diagnosis of breast cancer or a negative diagnosis.

In another aspect, the invention provides methods for detecting at least one protein marker in a sample, wherein the marker is selected from: Marker BrH1: 6850 ± 14 Da; Marker BrH2: 8565 ± 17 Da; Marker BrH3: 8920 ± 18 Da; Marker BrH4: 11180 ± 22 Da; Marker BrH5: 15220 ± 30 Da; Marker BrH6: 106080 ± 530 Da; Marker BrH7: 117600 ± 590 Da; Marker BrM1: 2804 ± 6 Da;Marker BrM2: 3390 ± 7 Da; Marker BrM3: 5890 ± 12 Da; Marker BrM4: 11900 ± 24 Da; and Marker BrM5: 51000 ± 260 Da; wherein the method comprises detecting the marker or markers by gas phase ion spectrometry.

In one embodiment, the methods comprise detecting the marker or markers by laser desorption/ionization mass spectrometry.

In another embodiment, the methods further comprise comparing the amount of the detected marker or markers to a control.

In another embodiment, the methods comprise (a) generating data on the sample with the mass spectrometer indicating intensity of signal for mass/charge ratio; (b) transforming the data into computer-readable form; and (c) operating a computer and executing an algorithm that detects signal in the computer-readable data representing the marker or markers.

In another embodiment, laser desorption/ionization mass spectrometry used to detect a marker or markers comprises (a) providing a substrate comprising an adsorbent attached thereto; (b) contacting the sample with the adsorbent; and (c) desorbing and ionizing the marker or markers from the substrate and detecting the desorbed/ionized marker or markers with the mass spectrometer.

In another embodiment, the methods further comprise sample preparation methods which can improve detection resolution of the markers. For example, the sample preparation includes fractionating a sample by size exclusion chromatography and by anion exchange chromatography, and collecting a sample fraction that includes the marker or markers, and performing gas phase ion spectrometry. In another example, the sample preparation includes removing serum albumin from a sample and collecting a sample fraction that includes the marker or markers, and performing gas phase ion spectrometry. In another example, the sample preparation includes fractionating a sample by anion exchange chromatography and collecting a sample fraction that includes the marker or markers and performing gas phase ion spectrometry. In another example, the sample preparation includes contacting a sample with a series of adsorbents (*e.g.*, cationic, lectin, metal chelating, *etc.*) in tandem to capture markers and performing gas phase ion spectrometry at each adsorbent to detect marker or markers. In another example, the sample preparation includes separating biomolecules in a sample by a gel electrophoresis or high performance liquid chromatography ("HPLC") and obtaining a fraction suspected of comprising the marker or markers and performing gas phase ion spectrometry.

In another aspect, the invention provides methods for detecting at least one protein marker in a sample, wherein the marker is selected from: Marker BrH1: 6850 ± 14 Da; Marker BrH2: 8565 ± 17 Da; Marker BrH3: 8920 ± 18 Da; Marker BrH4: 11180 ± 22 Da; Marker BrH5: 15220 ± 30 Da; Marker BrH6: 106080 ± 530 Da; Marker BrH7: 117600 ± 590 Da; Marker BrM1: 2804 ± 6 Da; Marker BrM2: 3390 ± 7 Da; Marker

BrM3: 5890 ± 12 Da; Marker BrM4: 11900 ± 24 Da; and Marker BrM5: 51000 ± 260 Da; wherein the method comprises detecting the marker or markers by an immunoassay.

In another aspect, the invention provides purified proteins selected from: Marker BrH1: 6850 ± 14 Da; Marker BrH2: 8565 ± 17 Da; Marker BrH3: 8920 ± 18 Da; Marker BrH4: 11180 ± 22 Da; Marker BrH5: 15220 ± 30 Da; Marker BrH6: 106080 ± 530 Da; Marker BrH7: 117600 ± 590 Da; Marker BrM1: 2804 ± 6 Da; Marker BrM2: 3390 ± 7 Da; Marker BrM3: 5890 ± 12 Da; Marker BrM4: 11900 ± 24 Da; and Marker BrM5: 51000 ± 260 Da.

In another aspect, the invention provides kits comprising: (a) a substrate comprising an adsorbent attached thereto, wherein the adsorbent is capable of retaining at least one protein marker selected from: Marker BrH1: 6850 ± 14 Da; Marker BrH2: 8565 ± 17 Da; Marker BrH3: 8920 ± 18 Da; Marker BrH4: 11180 ± 22 Da; Marker BrH5: 15220 ± 30 Da; Marker BrH6: 106080 ± 530 Da; Marker BrH7: 117600 ± 590 Da; Marker BrM1: 2804 ± 6 Da; Marker BrM2: 3390 ± 7 Da; Marker BrM3: 5890 ± 12 Da; Marker BrM4: 11900 ± 24 Da; and Marker BrM5: 51000 ± 260 Da; and (b) instructions to detect the marker or markers by contacting a sample with the adsorbent and detecting the marker or markers retained by the adsorbent.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates a sample pre-fractionation protocol, wherein the sample is first pre-fractionated using the K-30 size-selection spin column and then further fractionated using the Q anion-exchange spin column, prior to gas phase ion spectrometry analysis.

Figure 2 illustrates a standard protocol, wherein the sample is pre-fractionated by removing serum albumin using a Cibacron blue column prior to gas phase ion spectrometry analysis.

Figure 3 illustrates a modified standard protocol, wherein the sample is pre-fractionated by removing serum albumin using an anti-HSA column prior to gas phase ion spectrometry analysis.

Figure 4 illustrates an ion exchange spin column protocol.

Figure 5 illustrates a tandem sequence extraction protocol.

Figure 6 illustrates a probe adapted for use with a gas phase ion spectrometer, wherein substrate 101 is in the form of a strip, upon which a plurality of discrete spots of adsorbents 102 is disposed.

Figure 7 illustrates breast cancer serum markers that are detected using a combination of size exclusion chromatography and anion exchange chromatography as shown in Figure 1.

Figures 8A, 8B and 8C illustrate the analysis of spectral view data using Spotfire Pro analysis database.

Figures 9A and 9B illustrate Spotfire Scatter Plot of markers BrM1 and BrM2, respectively.

Figures 10A and 10B illustrate a Spotfire Scatter Plot of marker BrM3 and a gel view of marker BrM3, respectively.

Figures 11A, 11B and 11C illustrate the detection of marker BrM4 under different conditions using the standard protocol. Figure 11A illustrates a Spotfire Scatter Plot of marker BrM4. Figure 11B illustrates a gel view of marker BrM4 detection when the probe was washed with wash solution no. 1 ("wash 1"). Figure 11C illustrates a gel view of marker BrM4 when the probe was washed with wash solution no. 2 ("Wash 2").

DEFINITIONS

Unless defined otherwise, all technical and scientific terms used herein have the meaning commonly understood by a person skilled in the art to which this invention belongs. The following references provide one of skill with a general definition of many of the terms used in this invention: Singleton *et al.*, *Dictionary of Microbiology and Molecular Biology* (2nd ed. 1994); *The Cambridge Dictionary of Science and Technology* (Walker ed., 1988); *The Glossary of Genetics*, 5th Ed., R. Rieger *et al.* (eds.), Springer Verlag (1991); and Hale & Marham, *The Harper Collins Dictionary of Biology* (1991). As used herein, the following terms have the meanings ascribed to them unless specified otherwise.

"Marker" in the context of the present invention refers to a polypeptide (of a particular apparent molecular weight) which is differentially present in a sample taken from patients having breast cancer as compared to a comparable sample taken from control subjects (*e.g.*, a person with a negative diagnosis or undetectable cancer, normal or healthy subject).

The phrase "differentially present" refers to differences in the quantity and/or the frequency of a marker present in a sample taken from patients having breast cancer as compared to a control subject. For examples, a marker can be a polypeptide which is present at an elevated level or at a decreased level in samples of breast cancer patients compared to samples of control subjects. Alternatively, a marker can be a polypeptide which is detected at a higher frequency or at a lower frequency in samples of breast cancer patients compared to samples of control subjects. A marker can be differentially present in terms of quantity, frequency or both.

A polypeptide is differentially present between the two samples if the amount of the polypeptide in one sample is statistically significantly different from the amount of the polypeptide in the other sample. For example, a polypeptide is differentially present between the two samples if it is present at least about 120%, at least about 130%, at least about 150%, at least about 180%, at least about 200%, at least about 300%, at least about 500%, at least about 700%, at least about 900%, or at least about 1000% greater than it is present in the other sample, or if it is detectable in one sample and not detectable in the other.

Alternatively or additionally, a polypeptide is differentially present between the two sets of samples if the frequency of detecting the polypeptide in the breast cancer patients' samples is statistically significantly higher or lower than in the control samples. For example, a polypeptide is differentially present between the two sets of samples if it is detected at least about 120%, at least about 130%, at least about 150%, at least about 180%, at least about 200%, at least about 300%, at least about 500%, at least about 700%, at least about 900%, or at least about 1000% more frequently or less frequently observed in one set of samples than the other set of samples.

"Diagnostic" means identifying the presence or nature of a pathologic condition. Diagnostic methods differ in their sensitivity and specificity. The "sensitivity" of a diagnostic assay is the percentage of diseased individuals who test positive (percent of "true positives"). Diseased individuals not detected by the assay are "false negatives." Subjects who are not diseased and who test negative in the assay, are termed "true negatives." The "specificity" of a diagnostic assay is 1 minus the false positive rate, where the "false positive" rate is defined as the proportion of those without the disease who test positive. While a particular diagnostic method may not provide a definitive diagnosis of a condition, it suffices if the method provides a positive indication that aids in diagnosis.

A "test amount" of a marker refers to an amount of a marker present in a sample being tested. A test amount can be either in absolute amount (*e.g.*, μg/ml) or a relative amount (*e.g.*, relative intensity of signals).

A "diagnostic amount" of a marker refers to an amount of a marker in a subject's sample that is consistent with a diagnosis of breast cancer. A diagnostic amount can be either in absolute amount (*e.g.*, μg/ml) or a relative amount (*e.g.*, relative intensity of signals).

A "control amount" of a marker can be any amount or a range of amount which is to be compared against a test amount of a marker. For example, a control amount of a marker can be the amount of a marker in a person without breast cancer. A control amount can be either in absolute amount (*e.g.*, μg/ml) or a relative amount (*e.g.*, relative intensity of signals).

"Probe" refers to a device that is removably insertable into a gas phase ion spectrometer and comprises a substrate having a surface for presenting a marker for detection. A probe can comprise a single substrate or a plurality of substrates. Terms such as ProteinChip®, ProteinChip® array, or chip are also used herein to refer to specific kinds of probes.

"Substrate" or "probe substrate" refers to a solid phase onto which an adsorbent can be provided (*e.g.*, by attachment, deposition, *etc.*).

"Adsorbent" refers to any material capable of adsorbing a marker. The term "adsorbent" is used herein to refer both to a single material ("monoplex adsorbent") (*e.g.*, a compound or functional group) to which the marker is exposed, and to a plurality of different materials ("multiplex adsorbent") to which the marker is exposed. The adsorbent materials in a multiplex adsorbent are referred to as "adsorbent species." For example, an addressable location on a probe substrate can comprise a multiplex adsorbent characterized by many different adsorbent species (*e.g.*, anion exchange materials, metal chelators, or antibodies), having different binding characteristics. Substrate material itself can also contribute to adsorbing a marker and may be considered part of an "adsorbent."

"Adsorption" or "retention" refers to the detectable binding between an absorbent and a marker either before or after washing with an eluant (selectivity threshold modifier) or a washing solution.

"Eluant" or "washing solution" refers to an agent that can be used to mediate adsorption of a marker to an adsorbent. Eluants and washing solutions are also

referred to as "selectivity threshold modifiers." Eluants and washing solutions can be used to wash and remove unbound materials from the probe substrate surface.

"Resolve," "resolution," or "resolution of marker" refers to the detection of at least one marker in a sample. Resolution includes the detection of a plurality of markers in a sample by separation and subsequent differential detection. Resolution does not require the complete separation of one or more markers from all other biomolecules in a mixture. Rather, any separation that allows the distinction between at least one marker and other biomolecules suffices.

"Gas phase ion spectrometer" refers to an apparatus that measures a parameter which can be translated into mass-to-charge ratios of ions formed when a sample is volatilized and ionized. Generally ions of interest bear a single charge, and mass-to-charge ratios are often simply referred to as mass. Gas phase ion spectrometers include, for example, mass spectrometers, ion mobility spectrometers, and total ion current measuring devices.

"Mass spectrometer" refers to a gas phase ion spectrometer that includes an inlet system, an ionization source, an ion optic assembly, a mass analyzer, and a detector.

"Laser desorption mass spectrometer" refers to a mass spectrometer which uses laser as means to desorb, volatilize, and ionize an analyte.

"Detect" refers to identifying the presence, absence or amount of the object to be detected.

The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers in which one or more amino acid residue is an analog or mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers. Polypeptides can be modified, *e.g.*, by the addition of carbohydrate residues to form glycoproteins. The terms "polypeptide," "peptide" and "protein" include glycoproteins, as well as non-glycoproteins.

"Detectable moiety" or a "label" refers to a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, or chemical means. For example, useful labels include ^{32}P, ^{35}S, fluorescent dyes, electron-dense reagents, enzymes (*e.g.*, as commonly used in an ELISA), biotin-streptavidin, dioxigenin, haptens and proteins for which antisera or monoclonal antibodies are available, or nucleic acid molecules with a sequence complementary to a target. The detectable moiety often

generates a measurable signal, such as a radioactive, chromogenic, or fluorescent signal, that can be used to quantify the amount of bound detectable moiety in a sample. Quantitation of the signal is achieved by, *e.g.*, scintillation counting, densitometry, or flow cytometry.

"Antibody" refers to a polypeptide ligand substantially encoded by an immunoglobulin gene or immunoglobulin genes, or fragments thereof, which specifically binds and recognizes an epitope (*e.g.*, an antigen). The recognized immunoglobulin genes include the kappa and lambda light chain constant region genes, the alpha, gamma, delta, epsilon and mu heavy chain constant region genes, and the myriad immunoglobulin variable region genes. Antibodies exist, *e.g.*, as intact immunoglobulins or as a number of well characterized fragments produced by digestion with various peptidases. This includes, *e.g.*, Fab' and $F(ab)'_2$ fragments. The term "antibody," as used herein, also includes antibody fragments either produced by the modification of whole antibodies or those synthesized de novo using recombinant DNA methodologies. It also includes polyclonal antibodies, monoclonal antibodies, chimeric antibodies, humanized antibodies, or single chain antibodies. "Fc" portion of an antibody refers to that portion of an immunoglobulin heavy chain that comprises one or more heavy chain constant region domains, CH_1, CH_2 and CH_3, but does not include the heavy chain variable region.

"Immunoassay" is an assay that uses an antibody to specifically bind an antigen (*e.g.*, a marker). The immunoassay is characterized by the use of specific binding properties of a particular antibody to isolate, target, and/or quantify the antigen.

The phrase "specifically (or selectively) binds" to an antibody or "specifically (or selectively) immunoreactive with," when referring to a protein or peptide, refers to a binding reaction that is determinative of the presence of the protein in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and do not substantially bind in a significant amount to other proteins present in the sample. Specific binding to an antibody under such conditions may require an antibody that is selected for its specificity for a particular protein. For example, polyclonal antibodies raised to marker BrM1 from specific species such as rat, mouse, or human can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with marker BrM1 and not with other proteins, except for polymorphic variants and alleles of marker BrM1. This selection may be achieved by subtracting out antibodies that cross-react with marker BrM1 molecules from other

species. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (*see, e.g.*, Harlow & Lane, *Antibodies, A Laboratory Manual* (1988), for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity). Typically a specific or selective reaction will be at least twice background signal or noise and more typically more than 10 to 100 times background.

"Energy absorbing molecule" or "EAM" refers to a molecule that absorbs energy from an ionization source in a mass spectrometer thereby aiding desorption of analyte, such as a marker, from a probe surface. Depending on the size and nature of the analyte, the energy absorbing molecule can be optionally used. Energy absorbing molecules used in MALDI are frequently referred to as "matrix." Cinnamic acid derivatives, sinapinic acid ("SPA"), cyano hydroxy cinnamic acid ("CHCA") and dihydroxybenzoic acid are frequently used as energy absorbing molecules in laser desorption of bioorganic molecules.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is based upon, in part, the discovery of protein markers that are differentially present in samples of breast cancer patients and control subjects, and the application of this discovery in methods and kits for aiding a breast cancer diagnosis. Some of these protein markers are found at an elevated level and/or more frequently in samples from breast cancer patients compared to a control (*e.g.*, women in whom breast cancer is undetectable). Accordingly, the amount of one or more markers found in a test sample compared to a control, or the mere detection of one or more markers in the test sample provides useful information regarding probability of whether a subject being tested has breast cancer or not.

The protein markers of the present invention have a number of other uses. For example, the markers can be used to screen for compounds that modulate the expression of the markers *in vitro* or *in vivo*, which compounds in turn may be useful in treating or preventing breast cancer in patients. In another example, markers can be used to monitor responses to certain treatments of breast cancer. In yet another example, the markers can be used in the heredity studies. For instance, certain markers may be genetically linked. This can be determined by, *e.g.*, analyzing samples from a population of breast cancer patients whose families have a history of breast cancer. The results can

then be compared with data obtained from, *e.g.*, breast cancer patients whose families do not have a history of breast cancer. The markers that are genetically linked may be used as a tool to determine if a subject whose family has a history of breast cancer is predisposed to having breast cancer.

I. Characterization of Markers

Two sets of markers were identified from blood serum samples of breast cancer patients using gas phase ion spectrometry. Each set was identified using different pre-fractionation protocols and different adsorbents as described in detail below.

A. Marker Set 1

A first set of markers was found by using a pre-fractionation protocol shown in Figure 1. As shown in Figure 1, a blood serum sample was first fractionated by size exclusion chromatography followed by anion exchange chromatography. Then, each fraction was applied to a substrate comprising a hydrophilic adsorbent (*i.e.*, SiO_2) and was tested by gas phase ion spectrometry to measure an apparent molecular weight of markers retained on the adsorbent. These markers were present at an elevated level in breast cancer patients' blood serum samples compared to control samples. Table 1 below shows apparent molecular weights of each marker and fractions in which the markers were found.

TABLE 1

Marker	App. M.W.	Fractions containing markers
BrH1	6850 ± 14 Da	Q1F1 fraction*, Q1F2 fraction
BrH2	8565 ± 17 Da	Q2F1 fraction*, Q1F2 fraction
BrH3	8920 ± 18 Da	Q1F1 fraction*, Q2F1 fraction
BrH4	11180 ± 22 Da	Q2F4 fraction*; Q1F4 fraction
BrH5	15220 ± 30 Da	Q1F1 fraction*
BrH6	106080 ± 530 Da	Q1F4 fraction*
BrH7	117600 ± 590 Da	Q1F4 fraction*

* notes a main fraction in which the markers were detected.

As shown in Table 1, the apparent molecular weight of each marker is represented as a range, because a molecular weight of a protein is typically resolved with confidence of about 0.2-0.5% variation by gas phase ion spectrometry. For example, an apparent molecular weight of a protein (having a molecular weight of less than 50,000

daltons) measured by gas phase ion spectrometry can vary about ± 0.2%. For a protein having a higher molecular weight (*e.g.*, 50,000 daltons or above), an apparent molecular weight of a protein measured by gas phase ion spectrometry can vary about ± 0.5%.

As shown in Table 1, each marker was typically found in one or more fractions when a blood serum sample was subject to combination of size exclusion chromatography and anion exchange chromatography. As shown in Figure 1, fractions "Q1" and "Q2" refer to two different fractions obtained by applying a serum sample to K-30 size-selection spin column. Q1 fraction contains relatively large proteins compared to Q2 fraction. Then each of Q1 fraction and Q2 fraction is separately applied to a Q anion-exchange spin column, and was eluted using a series of buffers having five different pH's. For example, F1 fraction was eluted with a buffer having pH of about 9. F2 fraction was eluted with a buffer having a pH of about 7. F3 fraction was eluted with a buffer having a pH of about 5. F4 fraction was eluted with a buffer having a pH of about 4. F5 fraction was eluted with a buffer having a pH of about 3.4, 1 M NaCl. Since a spin column only allows a crude separation of proteins according to their physical characteristics, some of the markers were typically found in more than in a single fraction.

The binding and elution characteristics of the markers provide information regarding markers' physical characteristics. For example, the first set of markers was bound to an anion exchange column and was subsequently eluted. Accordingly, the markers likely comprise negatively charged moieties. Markers that were eluted with an eluant having a lower pH (*e.g.*, F4 fraction) would likely be strongly negatively charged compared to markers that were eluted with an eluant having a higher pH (*e.g.*, F1 fraction). Moreover, the first set of markers was retained by a hydrophilic adsorbent (*see* the Example section). Accordingly, the first set of markers would likely comprise hydrophilic binding moieties.

B. Marker Set 2

A second set of markers was found by using a pre-fractionation protocol shown in Figures 2 or 3. Specifically a blood serum sample was applied to either a Cibacron blue column ("standard protocol" shown in Figure 2) or an anti-human serum albumin column ("modified standard protocol" shown in Figure 3). Then, each fraction was applied to a metal chelate adsorbent (comprising copper) and was tested by gas phase ion spectrometry to detect markers retained on the adsorbent. Some of the markers that were detected using a standard modified protocol were also found using different pre-

fractionation protocols (*e.g.*, the ion exchange spin column protocol shown in Figure 4 or the tandem sequence extraction protocol shown in Figure 5). Table 2 below shows apparent molecular weights of each marker, and pre-fractionation protocols and conditions that were used to detect markers.

TABLE 2

Marker	App. M.W.	Conditions
BrM1	2804 ± 6 Da	Standard Protocol (Cu^{++} adsorbent) Modified Std. Protocol (Cu^{++} adsorbent)
BrM2	3390 ± 7 Da	Standard Protocol (Cu^{++} adsorbent) Modified Std. Protocol (Cu^{++} adsorbent) Tandem Extraction (Lectin adsorbent)
BrM3	5890 ± 12 Da	Standard Protocol (Cu^{++} adsorbent) Modified Std. Protocol (Cu^{++} adsorbent)
BrM4	11900 ± 24 Da	Standard Protocol (Cu^{++} adsorbent) Modified Std. Protocol (Cu^{++} adsorbent) Ion-Exchange Spin Column (Q3; Cu^{++} adsorbent)
BrM5	51000 ± 260 Da	Standard Protocol (Cu^{++} adsorbent) Modified Std. Protocol (Cu^{++} adsorbent) Tandem Extraction (Cu^{++} adsorbent)

As shown in Table 2, each marker in Marker Set 2 can be obtained using various pre-fractionation protocols. For example, a standard protocol shown in Figure 2 can be used to pre-fractionate a blood serum sample and to detect all of the markers in Marker Set 2. A standard protocol uses a Cibacron blue column to remove serum albumin, along with other proteins (*e.g.*, kinases, dehydrogenases, and other enzymes requiring adenyl-containing cofactors, lipoproteins, blood coagulation factors, interferons, *etc.*). The serum albumin-removed sample was then applied to a substrate comprising a copper chelate adsorbent, and markers BrM1, BrM2, BrM3, BrM4 and BrM5 retained on the adsorbent were detected using gas phase ion spectrometry. Proteins with exposed histidine, tryptophan and/or cysteine typically bind to transitional metals immobilized on a substrate. Accordingly, the markers in Marker Set 2 would likely possess these metal binding amino acid residues that are exposed.

Markers BrM1, BrM2, BrM3 and BrM4 in Marker Set 2 were also detected using a modified standard protocol shown in Figure 3. A modified standard

protocol uses an anti-HSA column to remove albumin. The serum albumin-removed sample was applied to a substrate comprising a copper chelate adsorbent, and markers BrM1, BrM2, BrM3 and BrM4 retained on the adsorbent were detected using gas phase ion spectrometry.

Marker BrM4 is further characterized as follows. Marker BrM4 was contained in a fraction when the blood serum sample was subject to anion exchange (Q) spin column chromatography and when the column was eluted with a buffer comprising 100 mM Na citrate, pH 3. Marker was retained on a copper chelate adsorbent by SELDI mass spectrometry. Since the marker BrM4 was eluted in a pH 3 fraction from an anion exchange spin column, the marker would likely be a protein that is negatively charged at a neutral pH.

Marker BrM2 is further characterized as follows. The serum was subject to Cibacron blue column chromatography to remove serum albumin. A sample fraction (from which serum albumin was removed) was applied to a cationic adsorbent (*e.g.*, SAX2 ProteinChip®). The fraction that did not bind to the SAX2 ProteinChip® was collected and applied the wheat germ lectin adsorbent and was analyzed using SELDI mass spectrometry. From this fraction, marker BrM2 was detected. Since marker BrM2 does not bind to the cationic adsorbent (*i.e.*, SAX2 ProteinChip®) at a neutral pH, this implies that this marker may not have a negative net charge at a neutral pH. Since this marker binds to a wheat germ lectin adsorbent, marker BrM2 is likely to be glycosylated.

Marker BrM5 is further characterized as follows. The serum was subject to Cibacron blue column chromatography to remove serum albumin. A sample fraction (from which serum albumin is removed) was applied to a cationic adsorbent (SAX2 ProteinChip®). A second sample fraction that did not bind to the SAX2 ProteinChip® was collected and applied to the wheat germ lectin adsorbent. A third sample fraction of the sample that did not bind to the wheat germ lectin adsorbent was further applied to a copper chelate adsorbent and was analyzed using SELDI mass spectrometry. From this fraction, marker BrM5 was detected. Since marker BrM5 did not bind to the cationic adsorbent (SAX2 ProteinChip®) at a neutral pH, this implies that this marker may not have a negative net charge at a neutral pH. Since this marker also did not bind to the wheat germ lectin adsorbent, it is unlikely that marker BrM5 is highly glycosylated.

While the markers were initially identified from a blood serum sample, the markers may be present in other types of samples (*e.g.*, nipple aspirate, urine, saliva, *etc.*). Thus, samples from which the markers can be detected are not limited to a blood serum

sample. Moreover, while the markers were initially identified using the techniques described above, the detection of the markers are not limited by these techniques and other techniques (*e.g.*, immunoassays) can be used.

II. DETECTION OF MARKERS

In another aspect, the invention provides methods for detecting markers which are differentially present in the samples of a breast cancer patient and a control (*e.g.*, women in whom breast cancer is undetectable). The markers can be detected in a number of biological samples. The sample is preferably a biological fluid sample. Examples of a biological fluid sample useful in this invention include blood, blood serum, plasma, nipple aspirate, urine, tears, saliva, *etc.* Because all of the markers are found in blood serum, blood serum is a preferred sample source for embodiments of the invention.

Any suitable methods can be used to detect one or more of the markers described herein. For example, gas phase ion spectrometry can be used. This technique includes, *e.g.*, laser desorption/ionization mass spectrometry. Preferably, the sample is prepared prior to gas phase ion spectrometry, *e.g.*, pre-fractionation, two-dimensional gel chromatography, high performance liquid chromatography, *etc.* to assist detection of markers. Detection of markers can be achieved using methods other than gas phase ion spectrometry. For example, immunoassays can be used to detect the markers in a sample. These detection methods are described in detail below.

A. Detection by Gas Phase Ion Spectrometry

In a preferred embodiment, markers present in a sample are detected using gas phase ion spectrometry, and more preferably, using mass spectrometry. In one embodiment, matrix-assisted laser desorption/ionization ("MALDI") mass spectrometry can be used. In MALDI, the sample is typically quasi-purified to obtain a fraction that essentially consists of a marker using protein separation methods such as two-dimensional gel electrophoresis or high performance liquid chromatography (HPLC).

In another embodiment, surface-enhanced laser desorption/ionization mass spectrometry ("SELDI") can be used. SELDI uses a substrate comprising adsorbents to capture markers, which can then be directly desorbed and ionized from the substrate surface during mass spectrometry. Since the substrate surface in SELDI captures markers, a sample need not be quasi-purified as in MALDI. However, depending on the complexity of a sample and the type of adsorbents used, it may be desirable to prepare a sample to reduce its complexity prior to SELDI analysis.

Various sample preparation methods to assist detection of markers in a sample and gas phase ion spectrometry methods are described in detail below.

1. Preparation of a Sample Prior to Gas Phase Ion Spectrometry

Optionally, one or combination of methods described below can be used to prepare a sample to further assist detection and characterization of markers in a sample. For example, a sample can be pre-fractionated to provide a less complex sample prior to gas phase ion spectrometry analysis. For example, in a blood serum sample, serum albumin is present in a high quantity and may obscure analysis of markers. By removing serum albumin using a pre-fractionation protocol, markers in a sample can be readily detected and analyzed by gas phase ion spectrometry. Moreover, pre-fractionation protocols can provide additional information regarding physical and chemical characteristics of markers. For example, if a sample was pre-fractionated using an anion-exchange spin column, and if a marker is eluted at a certain pH, this elution characteristic provides information regarding binding properties of the marker. Other suitable sample preparation protocols will be apparent to one of skill in the art, and they can also be applied in embodiments of the present invention.

a) Size Exclusion Chromatography

In one embodiment, a sample can be pre-fractionated according to size of proteins in a sample using size exclusion chromatography. For a biological sample wherein the amount of sample available is small, preferably a size selection spin column is used. For example, Q anion-exchange spin column chromatography comprising a quaternary ammonium functional group can be used (*e.g.*, QAE Sepharose, QAE Cellulose, Pharmacia). Figure 1 illustrates an example of a size exclusion chromatography protocol and Example section IV provides details of the protocol. In general, the first fraction that is eluted from the column ("fraction 1") has the highest percentage of high molecular weight proteins; fraction 2 has a lower percentage of high molecular weight proteins; fraction 3 has even a lower percentage of high molecular weight proteins; fraction 4 has the lowest amount of large proteins; and so on. Each fraction can then be analyzed by gas phase ion spectrometry for the detection of markers.

b) Anion Exchange Chromatography

In another embodiment, a sample can be pre-fractionated by anion exchange chromatography. Anion exchange chromatography allows pre-fractionation of the proteins in a sample roughly according to their charge characteristics. For example, a

Q anion-exchange spin column can be used, and a sample can be sequentially eluted with eluants having different pH's (*see* Figure 1 and Example section IV). Anion exchange chromatography allows separation of biomolecules in a sample that are more negatively charged from other types of biomolecules. Proteins that are eluted with an eluant having a high pH is likely to be weakly negatively charged, and a fraction that is eluted with an eluant having a low pH is likely to be strongly negatively charged. Thus, in addition to reducing complexity of a sample, anion exchange chromatography separates proteins according to their binding characteristics.

c) Affinity Chromatography

In yet another embodiment, a sample can be pre-fractionated by removing proteins that are present in a high quantity or that may interfere with the detection of markers in a sample. For example, in a blood serum sample, serum albumin is present in a high quantity and may obscure the analysis of markers. Thus, a blood serum sample can be pre-fractionated by removing serum albumin. Serum albumin can be removed using a substrate that comprises adsorbents that specifically bind serum albumin. For example, a column which comprises, *e.g.*, Cibacron blue (which has a high affinity for serum albumin) or anti-serum albumin antibodies can be used (*see*, *e.g.*, Figures 2 and 3).

d) Sequential Extraction

In yet another embodiment, a sample can be fractionated using a sequential extraction protocol. In sequential extraction, a sample is exposed to a series of adsorbents to extract different types of biomolecules from a sample. For example, a sample is applied to a first adsorbent to extract certain proteins, and an eluant containing non-adsorbent proteins (*i.e.*, proteins that did not bind to the first adsorbent) is collected. Then, the eluant is exposed to a second adsorbent. This further extracts various proteins from the eluant. This second eluant is then exposed to a third adsorbent, and so on. An example of a sequential extraction protocol is shown in Figure 5. As shown in Figure 5, typically adsorbents in a sequential extraction protocol have different binding groups. For example, a first adsorbent comprises an anionic group, a second adsorbent comprises wheat germ lectin, a third adsorbent comprises a metal chelate group, and a fourth adsorbent comprises heparin. These adsorbents can extract biomolecules having different binding characteristics. Various permutations of the order of these or other adsorbents can be applied in a sequential extraction protocol.

Any suitable materials and methods can be used to perform sequential extraction of a sample. For example, a series of spin columns comprising different adsorbents can be used. In another example, a multi-well comprising different adsorbents at its bottom can be used. In another example, sequential extraction can be performed on a probe adapted for use in a gas phase ion spectrometer, wherein the probe surface comprises adsorbents for binding biomolecules. In this embodiment, the sample is applied to a first adsorbent on the probe, which is subsequently washed with an eluant. Markers that do not bind to the first adsorbent is removed with an eluant. The markers that are in the eluant can be applied to a second adsorbent on the probe, and so forth. The advantage of performing sequential extraction on a gas phase ion spectrometer probe is that markers that bind to various adsorbents at every stage of the sequential extraction protocol can be analyzed directly using a gas phase ion spectrometer.

e) Separation of Biomolecules by Gel Electrophoresis

In yet another embodiment, biomolecules in a sample can be separated by high-resolution electrophoresis, *e.g.*, one or two-dimensional gel electrophoresis. A fraction containing a marker can be isolated and further analyzed by gas phase ion spectrometry. Preferably, two-dimensional gel electrophoresis is used to generate two-dimensional array of spots of biomolecules, including one or more markers. *See, e.g.*, Jungblut and Thiede, *Mass Spectr. Rev.* 16:145-162 (1997).

The two-dimensional gel electrophoresis can be performed using methods known in the art. *See, e.g.*, Deutscher ed., *Methods In Enzymology* vol. 182. Typically, biomolecules in a sample are separated by, *e.g.*, isoelectric focusing, during which biomolecules in a sample are separated in a pH gradient until they reach a spot where their net charge is zero (*i.e.*, isoelectric point). This first separation step results in one-dimensional array of biomolecules. The biomolecules in one dimensional array is further separated using a technique generally distinct from that used in the first separation step. For example, in the second dimension, biomolecules separated by isoelectric focusing are further separated using a polyacrylamide gel, such as polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE). SDS-PAGE gel allows further separation based on molecular mass of biomolecules. Typically, two-dimensional gel electrophoresis can separate chemically different biomolecules in the molecular mass range from 1000-200,000 Da within complex mixtures.

Biomolecules in the two-dimensional array can be detected using any suitable methods known in the art. For example, biomolecules in a gel can be labeled or stained (*e.g.*, Coomassie Blue or silver staining). If gel electrophoresis generates spots that correspond to the molecular weight of one or more markers of the invention, the spot can be is further analyzed by gas phase ion spectrometry. For example, spots can be excised from the gel and analyzed by gas phase ion spectrometry. Alternatively, the gel containing biomolecules can be transferred to an inert membrane by applying an electric field. Then a spot on the membrane that approximately corresponds to the molecular weight of a marker can be analyzed by gas phase ion spectrometry. In gas phase ion spectrometry, the spots can be analyzed using any suitable techniques, such as MALDI or SELDI (*e.g.*, using ProteinChip® array) as described in detail below.

Prior to gas phase ion spectrometry analysis, it may be desirable to cleave biomolecules in the spot into smaller fragments using cleaving reagents, such as proteases (*e.g.*, trypin). The digestion of biomolecules into small fragments provides a mass fingerprint of the biomolecules in the spot, which can be used to determine the identity of markers if desired.

f) High Performance Liquid Chromatography

In yet another embodiment, high performance liquid chromatography (HPLC) can be used to separate a mixture of biomolecules in a sample based on their different physical properties, such as polarity, charge and size. HPLC instruments typically consist of a reservoir of mobile phase, a pump, an injector, a separation column, and a detector. Biomolecules in a sample are separated by injecting an aliquot of the sample onto the column. Different biomolecules in the mixture pass through the column at different rates due to differences in their partitioning behavior between the mobile liquid phase and the stationary phase. A fraction that corresponds to the molecular weight and/or physical properties of one or more markers can be collected. The fraction can then be analyzed by gas phase ion spectrometry to detect markers. For example, the spots can be analyzed using either MALDI or SELDI (*e.g.*, using ProteinChip® array) as described in detail below.

g) Modification of Marker Before Analysis

Optionally, a marker can be modified before analysis to improve its resolution or to determine its identity. For example, the markers may be subject to proteolytic digestion before analysis. Any protease can be used. Proteases, such as

trypsin, that are likely to cleave the markers into a discrete number of fragments are particularly useful. The fragments that result from digestion function as a fingerprint for the markers, thereby enabling their detection indirectly. This is particularly useful where there are markers with similar molecular masses that might be confused for the marker in question. Also, proteolytic fragmentation is useful for high molecular weight markers because smaller markers are more easily resolved by mass spectrometry. In another example, biomolecules can be modified to improve detection resolution. For instance, neuraminidase can be used to remove terminal sialic acid residues from glycoproteins to improve binding to an anionic adsorbent (*e.g.*, cationic exchange ProteinChip® arrays) and to improve detection resolution. In another example, the markers can be modified by the attachment of a tag of particular molecular weight that specifically bind to molecular markers, further distinguishing them. Optionally, after detecting such modified markers, the identity of the markers can be further determined by matching the physical and chemical characteristics of the modified markers in a protein database (*e.g.*, SwissProt).

2. Contacting a Sample with a Substrate for Gas Phase Ion Spectrometry Analysis

A sample or a sample that is prepared as described above can be contacted with a substrate. A substrate can be a probe that is adapted for use with a gas phase ion spectrometer. Alternatively, a substrate can be a separate material that can be placed onto a probe that is adapted for use with a gas phase ion spectrometer. For example, a substrate can be a solid phase, such as a polymeric, paramagnetic, latex or glass bead comprising, *e.g.*, a functional group for binding markers. The substrate can then be positioned onto a probe.

A probe can be in any suitable shape as long as it is adapted for use with a gas phase ion spectrometer (*e.g.*, removably insertable into a gas phase ion spectrometer). For example, the probe can be in the form of a strip, a plate, or a dish with a series of wells at predetermined addressable locations. The probe can also be shaped for use with inlet systems and detectors of a gas phase ion spectrometer. For example, the probe can be adapted for mounting in a horizontally and/or vertically translatable carriage that horizontally and/or vertically moves the probe to a successive position without requiring repositioning of the probe by hand.

The probe substrate can be made of any suitable material. For example, the probe substrate material can include, but is not limited to, insulating materials (*e.g.*,

glass such as silicon oxide, plastic, ceramic), semi-conducting materials (*e.g.*, silicon wafers), or electrically conducting materials (*e.g.*, metals, such as nickel, brass, steel, aluminum, gold, or electrically conductive polymers), organic polymers, biopolymers, or any combinations thereof. The probe substrate material can also be solid or porous. Probes suitable for use in embodiments of the invention are described in, *e.g.*, U.S. Patent No. 5,617,060 (Hutchens and Yip) and WO 98/59360 (Hutchens and Yip).

a) Analysis of Samples on an Inert Substrate

If complexity of a sample has been substantially reduced using the preparation methods described above, the sample can be contacted with any suitable substrate for gas phase ion spectrometry. For example, the substrate surface can be inert and need not comprise adsorbents for binding markers, since further separation of other biomolecules from markers is not necessary. In some embodiments, preferably a sample is prepared by two-dimensional gel electrophoresis or HPLC to obtain a fraction that contains markers prior to contacting the fraction with a substrate. Then the markers in the spot or fraction can be resolved using gas phase ion spectrometry (*e.g.*, traditional MALDI) without further fractionation.

Prior to gas phase ions spectrometry analysis, an energy absorbing molecule ("EAM") or a matrix material is typically applied to markers on the substrate surface. The energy absorbing molecules can assist absorption of energy from an energy source from a gas phase ion spectrometer, and can assist desorption of markers from the probe surface. Exemplary energy absorbing molecules include cinnamic acid derivatives, sinapinic acid ("SPA"), cyano hydroxy cinnamic acid ("CHCA") and dihydroxybenzoic acid. Other suitable energy absorbing molecules are known to those skilled in this art. *See, e.g.,* U.S. Patent 5,719,060 (Hutchens & Yip) for additional description of energy absorbing molecules.

The energy absorbing molecule and the sample containing markers can be contacted in any suitable manner. For example, an energy absorbing molecule is mixed with a sample containing markers, and the mixture is placed on the substrate surface, as in traditional MALDI process. In another example, an energy absorbing molecule can be placed on the substrate surface prior to contacting the substrate surface with a sample. In another example, a sample can be placed on the substrate surface prior to contacting the substrate surface with an energy absorbing molecule. Then the markers can be desorbed, ionized and detected as described in detail below.

b) Analysis of Samples on a Substrate Surface Comprising Adsorbents

In some embodiments, complexity of a sample can be further reduced using a substrate that comprises adsorbents capable of binding one or more markers. Adsorbents need not be biospecific for markers (*e.g.*, antibodies that specifically bind markers) as long as adsorbents have binding characteristics suitable for binding markers. For example, adsorbents can comprise a hydrophobic group, a hydrophilic group, a cationic group, an anionic group, a metal ion chelating group, lectin, heparin, or antibodies, or any combination thereof.

Examples of various types of adsorbents are well-known in the art. For instance, adsorbents comprising a hydrophobic group include matrices having aliphatic hydrocarbons, *e.g.*, C_1-C_{18} aliphatic hydrocarbons and matrices having aromatic hydrocarbon functional group such as phenyl groups. Adsorbents comprising a hydrophilic group include, *e.g.*, glass (*e.g.*, silicon oxide), or hydrophilic polymers such as polyethylene glycol, dextran, agarose, or cellulose. Adsorbents comprising a cationic group include, *e.g.*, matrices of secondary, tertiary or quaternary amines. Adsorbents comprising an anionic group include, *e.g.*, matrices of sulfate anions (SO_3^-) and matrices of carboxylate anions (COO^-) or phosphate anions (OPO_3^-). Adsorbents comprising metal chelating groups include, *e.g.*, organic molecules that have one or more electron donor groups which form coordinate covalent bonds with metal ions, such as copper, nickel, cobalt, zinc, iron, and other metal ions such as aluminum and calcium. Adsorbents comprising an antibody include, *e.g.*, an antibody that specifically binds to any one of the markers provided herein. In preferred embodiments, adsorbents are substantially similar to or the same as the adsorbents which were initially used to enrich and identify the markers from a blood serum sample.

The probes can be produced using any suitable methods depending on the selection of substrate materials and/or adsorbents. For example, a metal surface can be coated with silicon oxide, titanium oxide or gold, and the coated surface can be derivatized with, *e.g.*, a bifunctional linker to bind and attach an adsorbent. For example, one end of a bifunctional linker can covalently bind with a functional group on the surface and the other end can be further derivatized with groups that function as an adsorbent. In another example, a porous silicon surface generated from crystalline silicon can be chemically modified to include adsorbents for binding markers. In another

example, adsorbents can be formed directly on the substrate surface by *in situ* polymerizing a monomer solution which comprises, *e.g.*, substituted acrylamide monomers, substituted acrylate monomers, or derivatives thereof comprising a functional group of choice as an adsorbent. The polymerization of the monomer solution can provide hydrogel adsorbents with a greater capacity for binding biomolecules.

Adsorbents that bind the markers can be applied to the substrate in any suitable pattern (*e.g.*, continuous or discontinuous). For example, one or more adsorbents can be present on the substrate surface. If multiple types of adsorbents are used, the substrate surface can be coated such that one or more binding characteristics vary in one or two-dimensional gradient. If discontinuous, plural adsorbents can be on the substrate surface in predetermined addressable locations. The addressable locations can be arranged in any pattern, but are preferably in a regular pattern, such as lines, orthogonal arrays, or regular curves (*e.g.*, circles). For example, as shown in Figure 6, a probe can comprise discontinuous spots of adsorbents. Each addressable location may comprise the same or different adsorbent. The spots are "addressable" in that during mass spectrometry, an energy source, such as a laser, is directed to, or "addresses" each spot to desorb and ionize markers.

A sample is contacted with a substrate comprising an adsorbent in any suitable manner, *e.g.*, bathing, soaking, dipping, spraying, washing over, or pipetting, *etc.* Generally, a volume of sample containing from a few attomoles to 100 picomoles of marker in about 1 µl to 500 µl is sufficient for binding to the adsorbent. The sample can contact the probe substrate comprising an adsorbent for a period of time sufficient to allow the marker to bind to the adsorbent. Typically, the sample and the substrate comprising the adsorbent are contacted for a period of between about 30 seconds and about 12 hours, and preferably, between about 30 seconds and about 15 minutes. Typically, the sample is contacted to the probe substrate under ambient temperature and pressure conditions. For some samples, however, modified temperature (typically 4°C through 37°C) and pressure conditions can be desirable, which conditions are determinable by those skilled in the art.

After the substrate contacts the sample or sample solution, it is preferred that unbound materials on the substrate surface are washed out so that only the bound materials remain on the substrate surface. Washing a substrate surface can be accomplished by, *e.g.*, bathing, soaking, dipping, rinsing, spraying, or washing the substrate surface with an eluant. A microfluidics process is preferably used when an

eluant is introduced to small spots of adsorbents on the probe. Typically, the eluant can be at a temperature of between 0°C and 100°C, preferably between 4°C and 37°C. In some embodiments, washing unbound materials from the probe surface may not be necessary if markers bound on the probe surface can be resolved by gas phase ion spectrometry without a wash.

Any suitable eluants (*e.g.*, organic or aqueous) can be used to wash the substrate surface. Preferably, an aqueous solution is used. Exemplary aqueous solutions include a HEPES buffer, a Tris buffer, or a phosphate buffered saline, *etc.* To increase the wash stringency of the buffers, additives can be incorporated into the buffers. These include, but are limited to, ionic interaction modifier (both ionic strength and pH), water structure modifier, hydrophobic interaction modifier, chaotropic reagents, affinity interaction displacers. Specific examples of these additives can be found in, *e.g.*, PCT publication WO98/59360 (Hutchens and Yip). The selection of a particular eluant or eluant additives is dependent on other experimental conditions (*e.g.*, types of adsorbents used or markers to be detected), and can be determined by those of skill in the art.

Prior to desorption and ionization of biomolecules including markers from the probe surface, an energy absorbing molecule ("EAM") or a matrix material is typically applied to the substrate surface. The types of EAM and the methods for applying EAM is discussed above, and will not be repeated in this section.

3. Desorption/Ionization and Detection

Markers on the substrate surface can be desorbed and ionized using gas phase ion spectrometry. Any suitable gas phase ion spectrometers can be used as long as it allows markers on the substrate to be resolved. Preferably, gas phase ion spectrometers allow quantitation of markers.

In one embodiment, a gas phase ion spectrometer is a mass spectrometer. In a typical mass spectrometer, a substrate or a probe comprising markers on its surface is introduced into an inlet system of the mass spectrometer. The markers are then desorbed by a desorption source such as a laser, fast atom bombardment, high energy plasma, electrospray ionization, thermospray ionization, liquid secondary ion MS, field desorption, *etc.* The generated desorbed, volatilized species consist of preformed ions or neutrals which are ionized as a direct consequence of the desorption event. Generated ions are collected by an ion optic assembly, and then a mass analyzer disperses and analyzes the passing ions. The ions exiting the mass analyzer are detected by a detector.

The detector then translates information of the detected ions into mass-to-charge ratios. Detection of the presence of markers or other substances will typically involve detection of signal intensity. This, in turn, can reflect the quantity and character of markers bound to the substrate. Any of the components of a mass spectrometer (*e.g.*, a desorption source, a mass analyzer, a detector, *etc.*) can be combined with other suitable components described herein or others known in the art in embodiments of the invention.

Preferably, a laser desorption time-of-flight mass spectrometer is used in embodiments of the invention. In laser desorption mass spectrometry, a substrate or a probe comprising markers is introduced into an inlet system. The markers are desorbed and ionized into the gas phase by laser from the ionization source. The ions generated are collected by an ion optic assembly, and then in a time-of-flight mass analyzer, ions are accelerated through a short high voltage field and let drift into a high vacuum chamber. At the far end of the high vacuum chamber, the accelerated ions strike a sensitive detector surface at a different time. Since the time-of-flight is a function of the mass of the ions, the elapsed time between ion formation and ion detector impact can be used to identify the presence or absence of markers of specific mass to charge ratio.

In another embodiment, an ion mobility spectrometer can be used to detect markers. The principle of ion mobility spectrometry is based on different mobility of ions. Specifically, ions of a sample produced by ionization move at different rates, due to their difference in, *e.g.*, mass, charge, or shape, through a tube under the influence of an electric field. The ions (typically in the form of a current) are registered at the detector which can then be used to identify a marker or other substances in a sample. One advantage of ion mobility spectrometry is that it can operate at atmospheric pressure.

In yet another embodiment, a total ion current measuring device can be used to detect and characterize markers. This device can be used when the substrate has a only a single type of marker. When a single type of marker is on the substrate, the total current generated from the ionized marker reflects the quantity and other characteristics of the marker. The total ion current produced by the marker can then be compared to a control (*e.g.*, a total ion current of a known compound). The quantity or other characteristics of the marker can then be determined.

4. Analysis of Data

Data generated by desorption and detection of markers can be analyzed using any suitable means. In one embodiment, data is analyzed with the use of a

programmable digital computer. The computer program generally contains a readable medium that stores codes. Certain code can be devoted to memory that includes the location of each feature on a probe, the identity of the adsorbent at that feature and the elution conditions used to wash the adsorbent. The computer also contains code that receives as input, data on the strength of the signal at various molecular masses received from a particular addressable location on the probe. This data can indicate the number of markers detected, including the strength of the signal generated by each marker.

Data analysis can include the steps of determining signal strength (*e.g.*, height of peaks) of a marker detected and removing "outerliers" (data deviating from a predetermined statistical distribution). The observed peaks can be normalized, a process whereby the height of each peak relative to some reference is calculated. For example, a reference can be background noise generated by instrument and chemicals (*e.g.*, energy absorbing molecule) which is set as zero in the scale. Then the signal strength detected for each marker or other biomolecules can be displayed in the form of relative intensities in the scale desired (*e.g.*, 100). Alternatively, a standard (*e.g.*, a serum protein) may be admitted with the sample so that a peak from the standard can be used as a reference to calculate relative intensities of the signals observed for each marker or other markers detected.

The computer can transform the resulting data into various formats for displaying. In one format, referred to as "spectrum view or retentate map," a standard spectral view can be displayed, wherein the view depicts the quantity of marker reaching the detector at each particular molecular weight. In another format, referred to as "peak map," only the peak height and mass information are retained from the spectrum view, yielding a cleaner image and enabling markers with nearly identical molecular weights to be more easily seen. In yet another format, referred to as "gel view," each mass from the peak view can be converted into a grayscale image based on the height of each peak, resulting in an appearance similar to bands on electrophoretic gels. In yet another format, referred to as "3-D overlays," several spectra can be overlaid to study subtle changes in relative peak heights. In yet another format, referred to as "difference map view," two or more spectra can be compared, conveniently highlighting unique markers and markers which are up- or down-regulated between samples. Marker profiles (spectra) from any two samples may be compared visually. In yet another format, Spotfire Scatter Plot can be used, wherein markers that are detected are plotted as a dot in a plot, wherein one axis of the plot represents the apparent molecular of the markers detected and another axis

represents the signal intensity of markers detected. For each sample, markers that are detected and the amount of markers present in the sample can be saved in a computer readable medium. This data can then be compared to a control (*e.g.*, a profile or quantity of markers detected in control, *e.g.*, women in whom breast cancer is undetectable).

B. Detection by Immunoassay

In another embodiment, an immunoassay can be used to detect and analyze markers in a sample. This method comprises: (a) providing an antibody that specifically binds to a marker; (b) contacting a sample with the antibody; and (c) detecting the presence of a complex of the antibody bound to the marker in the sample.

To prepare an antibody that specifically binds to a marker, purified markers or their nucleic acid sequences can be used. Nucleic acid and amino acid sequences for markers can be obtained by further characterization of these markers. For example, each marker can be peptide mapped with a number of enzymes (*e.g.*, trypsin, V8 protease, *etc.*). The molecular weights of digestion fragments from each marker can be used to search the databases, such as SwissProt database, for sequences that will match the molecular weights of digestion fragments generated by various enzymes. Using this method, the nucleic acid and amino acid sequences of other markers can be identified if these markers are known proteins in the databases.

Alternatively, the proteins can be sequenced using protein ladder sequencing. Protein ladders can be generated by, for example, fragmenting the molecules and subjecting fragments to enzymatic digestion or other methods that sequentially remove a single amino acid from the end of the fragment. Methods of preparing protein ladders are described, for example, in International Publication WO 93/24834 (Chait *et al.*) and United States Patent 5,792,664 (Chait *et al.*). The ladder is then analyzed by mass spectrometry. The difference in the masses of the ladder fragments identify the amino acid removed from the end of the molecule.

If the markers are not known proteins in the databases, nucleic acid and amino acid sequences can be determined with knowledge of even a portion of the amino acid sequence of the marker. For example, degenerate probes can be made based on the N-terminal amino acid sequence of the marker. These probes can then be used to screen a genomic or cDNA library created from a sample from which a marker was initially detected. The positive clones can be identified, amplified, and their recombinant DNA sequences can be subcloned using techniques which are well known. *See, e.g., Current*

Protocols for Molecular Biology (Ausubel *et al.*, Green Publishing Assoc. and Wiley-Interscience 1989) *and Molecular Cloning: A Laboratory Manual*, 3rd Ed. (Sambrook *et al.*, Cold Spring Harbor Laboratory, NY 2001).

Using the purified markers or their nucleic acid sequences, antibodies that specifically bind to a marker can be prepared using any suitable methods known in the art. *See, e.g.*, Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *Antibodies: A Laboratory Manual* (1988); Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986); and Kohler & Milstein, *Nature* 256:495-497 (1975). Such techniques include, but are not limited to, antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors, as well as preparation of polyclonal and monoclonal antibodies by immunizing rabbits or mice (*see, e.g.*, Huse *et al.*, *Science* 246:1275-1281 (1989); Ward *et al.*, *Nature* 341:544-546 (1989)).

After the antibody is provided, a marker can be detected and/or quantified using any of suitable immunological binding assays known in the art (*see, e.g.*, U.S. Patent Nos. 4,366,241; 4,376,110; 4,517,288; and 4,837,168). Useful assays include, for example, an enzyme immune assay (EIA) such as enzyme-linked immunosorbent assay (ELISA), a radioimmune assay (RIA), a Western blot assay, or a slot blot assay. These methods are also described in, *e.g.*, *Methods in Cell Biology: Antibodies in Cell Biology*, volume 37 (Asai, ed. 1993); *Basic and Clinical Immunology* (Stites & Terr, eds., 7th ed. 1991); and Harlow & Lane, *supra*.

Generally, a sample obtained from a subject can be contacted with the antibody that specifically binds the marker. Optionally, the antibody can be fixed to a solid support to facilitate washing and subsequent isolation of the complex, prior to contacting the antibody with a sample. Examples of solid supports include glass or plastic in the form of, *e.g.*, a microtiter plate, a stick, a bead, or a microbead. Antibodies can also be attached to a probe substrate or ProteinChip® array described above. The sample is preferably a biological fluid sample taken from a subject. Examples of biological fluid samples include blood, serum, plasma, nipple aspirate, urine, tears, saliva *etc.* In a preferred embodiment, the biological fluid comprises blood serum. The sample can be diluted with a suitable eluant before contacting the sample to the antibody.

After incubating the sample with antibodies, the mixture is washed and the antibody-marker complex formed can be detected. This can be accomplished by incubating the washed mixture with a detection reagent. This detection reagent may be,

e.g., a second antibody which is labeled with a detectable label. Exemplary detectable labels include magnetic beads (*e.g.*, DYNABEADS™), fluorescent dyes, radiolabels, enzymes (*e.g.*, horse radish peroxide, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic beads. Alternatively, the marker in the sample can be detected using an indirect assay, wherein, for example, a second, labeled antibody is used to detect bound marker-specific antibody, and/or in a competition or inhibition assay wherein, for example, a monoclonal antibody which binds to a distinct epitope of the marker is incubated simultaneously with the mixture.

Throughout the assays, incubation and/or washing steps may be required after each combination of reagents. Incubation steps can vary from about 5 seconds to several hours, preferably from about 5 minutes to about 24 hours. However, the incubation time will depend upon the assay format, marker, volume of solution, concentrations and the like. Usually the assays will be carried out at ambient temperature, although they can be conducted over a range of temperatures, such as 10°C to 40°C.

Immunoassays can be used to determine presence or absence of a marker in a sample as well as the quantity of a marker in a sample. First, a test amount of a marker in a sample can be detected using the immunoassay methods described above. If a marker is present in the sample, it will form an antibody-marker complex with an antibody that specifically binds the marker under suitable incubation conditions described above. The amount of an antibody-marker complex can be determined by comparing to a standard. A standard can be, *e.g.*, a known compound or another protein known to be present in a sample. As noted above, the test amount of marker need not be measured in absolute units, as long as the unit of measurement can be compared to a control.

The methods for detecting these markers in a sample have many applications. For example, one or more markers can be measured to aid breast cancer diagnosis or prognosis. In another example, the methods for detection of the markers can be used to monitor responses in a subject to cancer treatment. In another example, the methods for detecting markers can be used to assay for and to identify compounds that modulate expression of these markers *in vivo* or *in vitro*.

III. DIAGNOSIS OF BREAST CANCER

In another aspect, the invention provides methods for aiding a breast cancer diagnosis using one or more markers in Marker Set 1 or Marker Set 2. These markers can be used alone, in combination with other markers in either set, or with entirely different markers (*e.g.*, neu oncogene product) in aiding breast cancer diagnosis. The markers in Marker Set 1 and Marker Set 2 are differentially present in samples of a breast cancer patient and a normal subject in whom breast cancer is undetectable. For example, some of the markers are expressed at an elevated level and/or are present at a higher frequency in breast cancer patients than in normal subjects. Therefore, detection of one or more of these markers in a person would provide useful information regarding the probability that the person may have breast cancer.

Accordingly, embodiments of the invention include methods for aiding a breast cancer diagnosis, wherein the method comprises: (a) detecting at least one marker in a sample, wherein the marker is selected from Marker BrH1: 6850 ± 14 Da; Marker BrH2: 8565 ± 17 Da; Marker BrH3: 8920 ± 18 Da; Marker BrH4: 11180 ± 22 Da; Marker BrH5: 15220 ± 30 Da; Marker BrH6: 106080 ± 530 Da; Marker BrH7: 117600 ± 590 Da; Marker BrM1: 2804 ± 6 Da; Marker BrM2: 3390 ± 7 Da; Marker BrM3: 5890 ± 12 Da; Marker BrM4: 11900 ± 24 Da; and Marker BrM5: 51000 ± 260 Da; and (b) correlating the detection of the marker or markers with a probable diagnosis of breast cancer. The correlation may take into account the amount of the marker or markers in the sample compared to a control amount of the marker or markers (*e.g.*, in normal subjects in whom breast cancer is undetectable). The correlation may take into account the presence or absence of the markers in a test sample and the frequency of detection of the same markers in a control. The correlation may take into account both of such factors to facilitate determination of whether a subject has a breast cancer or not.

Any suitable samples can be obtained from a subject to detect markers. Preferably, a sample is a blood serum sample from the subject. If desired, the sample can be prepared as described above to enhance detectability of the markers. For example, to increase the detectability of markers BrH1, BrH2, BrH3, BrH4, BrH5, BrH6 and BrH7, a blood serum sample from the subject can be preferably fractionated by, *e.g.*, size exclusion chromatography and anion exchange chromatography. In another example, if the detection of markers BrM1, BrM2, BrM3, BrM4 and BrM5 is desired, then the sample is preferably pre-fractionated to remove serum albumin. In another example, the

sample can be pre-fractionated by anion exchange chromatography to increase detectability of marker BrH4. In yet another example, if the detection of markers BrM2 is desired, then the sample is preferably pre-fractionated to remove serum albumin and is subject to a tandem extraction protocol as describe above. Sample preparations, such as pre-fractionation protocols, is optional and may not necessary to enhance detectability of markers depending on the methods of detection used. For example, sample preparation may be unnecessary if antibodies that specifically bind markers are used to detect the presence of markers in a sample.

Any suitable method can be used to detect a marker or markers in a sample. For example, gas phase ion spectrometry or an immunoassay can be used as described above. Using these methods, one or more markers can be detected. Preferably, a sample is tested for the presence of a plurality of markers. Detecting the presence of a plurality of markers, rather than a single marker alone, would provide more information for the diagnotician. Specifically, the detection of a plurality of markers in a sample would increase the percentage of true positive and true negative diagnoses and would decrease the percentage of false positive or false negative diagnoses.

The detection of the marker or markers is then correlated with a probable diagnosis of breast cancer. In some embodiments, the detection of the mere presence or absence of a marker, without quantifying the amount of marker, is useful and can be correlated with a probable diagnosis of breast cancer. For example, markers BrH1, BrH3, BrH5, BrH6 and BrH7 are more frequently detected in breast cancer patients than in normal subjects (*see* Figure 7). In particular, markers BrH6 and BrH7 are four or three times, respectively, more frequently detected in blood serum samples of breast cancer patients than in blood serum samples of normal subjects. Thus, a mere detection of one or more of these markers in a subject being tested indicates that the subject has a higher probability of having a breast cancer.

In other embodiments, the detection of markers can involve quantifying the markers to correlate the detection of markers with a probable diagnosis of breast cancer. For example, markers BrH1, BrH2, BrH3, BrH4, BrH5 and BrH6 are present at a higher quantity in blood serum samples of breast cancer patients than in blood serum samples of normal subjects (*see* Figure 7). In particular, Marker BrH3 is present at least about 9.8 fold higher on average in blood serum samples of breast cancer patients than in blood serum samples of normal subjects. Thus, if the amount of the markers detected in a

subject being tested is higher compared to a control amount, then the subject being tested has a higher probability of having a breast cancer.

An analysis of the data shows that detection of any one of markers BrH1, BrH3, BrH5, BrH6 or BrH7 is not highly correlated with a positive diagnosis of breast cancer. However, the chance of a positive diagnosis increases significantly with the detection of any two, three, four or all of these markers. Furthermore, the failure to detect any one, two or any three of these markers also is not highly correlated with a negative diagnosis of breast cancer. However, the failure to detect any four or all five of these markers is highly correlated with a negative diagnosis of breast cancer.

When the markers are quantified, it can be compared to a control. A control can be, *e.g.*, the average or median amount of marker present in comparable samples of normal subjects in whom breast cancer is undetectable. The control amount is measured under the same or substantially similar experimental conditions as in measuring the test amount. For example, if a test sample is obtained from a subject's blood serum sample and a marker is detected using a particular probe, then a control amount of the marker is preferably determined from a serum sample of a patient using the same probe. It is preferred that the control amount of marker is determined based upon a significant number of samples from normal subjects who do not have breast cancer so that it reflects variations of the marker amounts in that population.

Data generated by mass spectrometry can then be analyzed by a computer software. The software can comprise code that converts signal from the mass spectrometer into computer readable form. The software also can include code that applies an algorithm to the analysis of the signal to determine whether the signal represents a "peak" in the signal corresponding to a marker of this invention, or other useful markers. The software also can include code that executes an algorithm that compares signal from a test sample to a typical signal characteristic of "normal" and breast cancer and determines the closeness of fit between the two signals. The software also can include code indicating which the test sample is closest to, thereby providing a probable diagnosis.

IV. KITS

In yet another aspect, the invention provides kits for aiding a diagnosis of breast cancer, wherein the kits can be used to detect the markers of the present invention. For example, the kits can be used to detect any one or more of the markers described

herein, which markers are differentially present in samples of a breast cancer patient and normal subjects. The kits of the invention have many applications. For example, the kits can be used to differentiate if a subject has breast cancer or has a negative diagnosis, thus aiding a breast cancer diagnosis. In another example, the kits can be used to identify compounds that modulate expression of one or more of the markers in *in vitro* or *in vivo* animal models for breast cancer.

In one embodiment, a kit comprises: (a) a substrate comprising an adsorbent thereon, wherein the adsorbent is suitable for binding a marker, and (b) instructions to detect the marker or markers by contacting a sample with the adsorbent and detecting the marker or markers retained by the adsorbent. In some embodiments, the kit may comprise an eluant (as an alternative or in combination with instructions) or instructions for making an eluant, wherein the combination of the adsorbent and the eluant allows detection of the markers using gas phase ion spectrometry. Such kits can be prepared from the materials described above, and the previous discussion of these materials (*e.g.*, probe substrates, adsorbents, washing solutions, *etc.*) is fully applicable to this section and will not be repeated.

In another embodiment, the kit may comprise a first substrate comprising an adsorbent thereon (*e.g.*, a particle functionalized with an adsorbent) and a second substrate onto which the first substrate can be positioned to form a probe which is removably insertable into a gas phase ion spectrometer. In other embodiments, the kit may comprise a single substrate which is in the form of a removably insertable probe with adsorbents on the substrate. In yet another embodiment, the kit may further comprise a pre-fractionation spin column (*e.g.*, Cibacron Blue column, anti-HSA column, K-30 size exclusion column, Q-anion exchange spin column, *etc.*).

Optionally, the kit can further comprise instructions for suitable operational parameters in the form of a label or a separate insert. For example, the kit may have standard instructions informing a consumer how to wash the probe after a sample of blood serum is contacted on the probe. In another example, the kit may have instructions for pre-fractionating a sample to reduce complexity of proteins in the sample.

In another embodiment, a kit comprises (a) an antibody that specifically binds to a marker; and (b) a detection reagent. Such kits can be prepared from the materials described above, and the previous discussion regarding the materials (*e.g.*, antibodies, detection reagents, immobilized supports, *etc.*) is fully applicable to this section and will not be repeated. Optionally, the kit may further comprise pre-

fractionation spin columns. In some embodiments, the kit may further comprise instructions for suitable operation parameters in the form of a label or a separate insert.

Optionally, the kit may further comprise a standard or control information so that the test sample can be compared with the control information standard to determine if the test amount of a marker detected in a sample is a diagnostic amount consistent with a diagnosis of breast cancer.

EXAMPLES

The following examples are offered by way of illustration, not by way of limitation. Illustrated below are the probe preparation protocols, the sample preparation protocols, and the identification of markers that have positive or negative correlations with breast cancer. While the sample protocols have been developed with human blood serum samples, the same general experimental set-up may be used for suitable samples to detect markers.

I. THE PROBE PREPARATION PROTOCOL

The examples of useful chips in the materials and methods of the invention include, but are not limited to, Normal Phase ProteinChip®, SAX2 ProteinChip®, IMAC3 Cu^{++} ProteinChip® (also referred to as Cu(II) chip), and Wheat Germ Lectin ProteinChip®, all available from Ciphergen Biosystems, Inc. (Fremont, CA).

A. Normal Phase ProteinChip® (silicon oxide surface)

Normal Phase ProteinChip® contain a SiO_2 surface for general binding of proteins. Hydrophilic SiO_2 surface binds proteins through electrostatic and dipole-dipole interactions as well as hydrogen binding. Proteins with hydrophilic and charged surface amino acids such as serine, threonine and lysine bind well to Normal Phase ProteinChip®. Binding typically occurs in aqueous buffers with a water wash prior to analysis.

Normal Phase ProteinChip® can be produced as follows. The surface of a metal substrate is conditioned by etching via laser (*e.g.*, Quantred Company, Galaxy model, ND-YAG Laser, using emission line of 1.064 nm, power of 30-35 watts with a laser spot size of 0.005 inches, the laser source to surface distance of 12-14 inches; with a rate of scan of about 25 per mm per second). Then the etched surface of the metal substrate is coated with a glass coating (SiO_2). Silicon oxide can be applied to the surface by any of a number of well known methods. These methods include, for example, vapor

deposition, *e.g.*, sputter coating. A preferred thickness for such a probe is about 9000 Angstroms.

Normal Phase ProteinChip® can bind markers BrH1, BrH2, BrH3, BrH4, BrH5, BrH6 and BrH7 as further described below.

B. SAX-2 ProteinChip® (Strong anionic exchanger, cationic surface)

SAX-2 ProteinChip® is a strong anion exchange array with a high capacity quaternary ammonium surface to bind anionic proteins. Anionic arrays bind proteins through electrostatic interaction of negatively charged amino acids such as aspartic acid and glutamic acid. Binding typically occurs at high pH with low salt, and binding decreases as pH decreases and salt concentration increases.

SAX-2 ProteinChip® can be produced as follows. The surface of the metal substrate is conditioned and coated with a glass coating as described above. 3-(Methacryloylamino)propyl trimethylammonium chloride (15.0 wt%) and N,N'-methylenebisacrylamide (0.4 wt%) are photo-polymerized using (-)-riboflavin (0.01 wt%) as a photo-initiator and ammonium persulfate (0.2 wt%) as an accelerant. The monomer solution is deposited onto a rough etched, glass coated substrate (0.4 μL, twice) and is irradiated for 5 minutes with a near UV exposure system (Hg short arc lamp, 20 mW/cm^2 at 365 nm). The surface is washed with a solution of sodium chloride (1 M), and then the surface is washed twice with deionized water.

C. IMAC3 ProtenChip® (Immobilized Metal Affinity Capture, nitrilotriacetic acid on surface)

IMAC3 ProteinChip® contains a surface for high-capacity metal binding and subsequent affinity capture of proteins with metal binding residues. Immobilized metal affinity capture arrays bind proteins and peptides which have affinity for metals; proteins with exposed histidine, tryptophan and/or cysteine typically bind to metals immobilized on these chip surfaces. Binding typically occurs under pH 6-8 and high salt, and binding decreases as the concentration of imidizole and glycine increase.

IMAC3 ProteinChip® can be produced as follows. The surface of the metal substrate is conditioned and coated with a glass coating as described above. 5-Methacylamido-2-(N,N-biscarboxymethaylamino)pentanoic acid (7.5 wt%), Acryloyltri-(hydroxymethyl)methylamine (7.5 wt%) and N,N'-methylenebisacrylamide (0.4 wt%) are photo-polymerized using-(-)riboflavin (0.02 wt%) as a photo-initiator. The monomer solution is deposited onto a rough etched, glass coated substrate (0.4 mL, twice) and

irradiated for 5 minutes with a near UV exposure system (Hg short arc lamp, 20 mW/cm2 at 365 nm). The surface is washed with a solution of sodium chloride (1 M) and then washed twice with deionized water.

IMAC3 ProteinChip® with Cu(II) can be activated with copper metal ions as follows. The surface is treated with a solution of 50 mM Cu sulfate (5 μL/spot) and mixed on a mixer for about 5 minutes. After removing the solution, the surface was rinsed with water. Then 5 μL of 0.1 M sodium acetate, pH 4, was added as a wash for 5 minutes, and this wash was repeated. Finally, the spot was equilibrated in PBS. This chip is also referred to IMAC3-Cu^{++} chip or Cu(II) chip.

IMAC3 ProteinChip® can bind markers BrM1, BrM2, BrM3, BrM4 and BrM5 as further described below.

D. Wheat Germ Lectin ProteinChip®

Wheat Germ Lectin ProteinChip® can be produced as follows. Wheat Germ Lectin ProteinChip® can be made by using a SELDI pre-activated probes (*e.g.*, ProteinChip® PS1 or PS2. ProteinChip® PS1 contains a carbonyldiimidazole surface which covalently reacts with amine groups. ProteinChip® PS2 contains an epoxy surface which covalently reacts with amine and thiol groups). DNA and proteins, including antibodies, can be immobilized on the PS1 and PS2 surfaces.

After placing the SELDI pre-activated ProteinChip® on a flat clean surface, 2 μL of wheat germ lectin (Sigma, 1 mg/ml solution 0.1 M sodium bicarbonate, pH 7.8) was added onto each spot of ProteinChip® array. The chip was incubated with wheat germ lectin mixing for 2-18 hours (4°C or room temperature, humid chamber). The remaining reagents were removed from the spots using a pipet. Residual active sites on the spots were blocked by adding 1 ml of 1 M ethanolamine, pH 8 over the entire chip, and then the chip was incubated in a humid chamber (room temperature 30 min). The chip was then washed twice with 1% Triton X-100 in PBS. The chip was submerged in about 10 ml of wash solution in a 15 ml conical plastic tube, and was shaken on benchtop shaker for 5 minutes. The chip was washed with 0.5 M NaCl in 0.1 M sodium acetate, pH 4.0. The chip was also washed with 0.5 M NaCl in 0.1 M TrisHCl, pH 8.0. The chip was rinsed with PBS, and then the chip was covered with 50 mM HEPES and stored at 4 °C until ready to use.

Wheat Germ Lectin ProteinChip® can bind marker BrM2 as further described below.

II. SERUM SAMPLE PROFILING ON ProteinChip®

A sample can be analyzed using a number of different chips. Preferably, "a multi-well bioprocessor" is used to analyze a number of samples simultaneously. If desired, each sample is washed with two or more different washes per chip type (*e.g.*, wash 1 and wash 2). These washes vary depending on the surface chemistry of the chip. Each sample can be analyzed using two or more different types of EAM (*e.g.*, sinapinic acid ("SPA") and cyano hydroxy cinnamic acid ("CHCA")). Thus, each sample can be analyzed under various conditions in order to maximize the diversity of proteins, including markers, detected. Samples can also be analyzed in multiple sets to minimize the impact of experimental variability. The protocols described below are merely exemplary and any variation thereof would be readily apparent to one of skill in the art. Accordingly, embodiments of the invention are not limited to the protocols described below.

A. Protein Profiling on SAX2 ProteinChip® or on Wheat Germ Lectin ProteinChip® Array

1. Put SAX2 or Wheat Germ Lectin chips in bioprocessor. Attach the top securely.
2. Add 200 µL 50 mM HEPES, pH 7.4 to each well.
3. Mix, 250 rpm on platform shaker, 5 min, RT.
4. Discard buffer.
5. Repeat steps 2-3.
6. To albumin-depleted serum samples: Dilute 10-fold in 50 mM HEPES, pH 7.4.
7. Add 50 µL diluted serum to each well.
8. Mix (250 rpm on a platform shaker) at room temperature for 15 min.
9. Remove the samples from SAX2 chips.
    a. Add 200 µL of 50 mM HEPES pH 7.4 (Wash 1); or
    b. Add 200 µL of 1 M urea 0.125% CHAPS, 0.25 M NaCl, 50 mM HEPES pH 7.4 (Wash 2).
10. Mix (250 rpm on a platform shaker) at room temperature for 5 min.
11. Remove the washes from the spots.
    a. Add fresh 200 µL of 50 mM HEPES (Wash 1); or

b. Add fresh 200 µL of 1 M urea 0.125% CHAPS, 0.25 M NaCl, 50 mM HEPES (Wash 2).

12. Mix vigorously (250 rpm on a platform shaker) at room temperature for 5 min.

13. Repeat buffer wash one more time.

14. Wash chips 3 times with water by filling the wells and emptying.

15. Remove chips from bioprocessor.

16. Rinse chips with deionized H_2O.

17. Air dry chips.

18. Apply pap pen if it has come off during the procedure.

19. Add 0.5 µL of SPA or CHCA to chips two times (air dry spots between additions).

B. Protein Profiling on IMAC3-Cu^{++} ProteinChip® Array

1. Add 10 µL of 100 mM $CuSO_4$ to each spot on IMAC3 chips.

2. Mix for 5 min, RT.

3. Rinse chips with water.

4. Wash each spot with 10 µL 100 mM sodium acetate pH 4.0 on a shaker for 5 min, RT.

5. Rinse chips with water.

6. Put chips in Bioprocessor. Attach the top securely.

7. Add 200 µL PBS buffer pH 7.2 to each spot.

8. Mix (250 rpm on a platform shaker) at room temperature for 5 min. Discard buffer.

9. Repeat wash with fresh PBS.

10. Dilute the albumin depleted serum sample 10 fold in PBS.

11. Add 50 µL diluted serum to all spots.

12. Mix vigorously (250 rpm on a platform shaker) at room temperature for 15 min.

13. Remove the samples from chips.

a. Add 200 µL of PBS (Wash 1) to chips; or

b. Add 200 µL of 1 M urea 0.125% CHAPS, 0.5 M NaCl, 100 mM acetate, pH 4.5 (Wash 2) to chips.

14. Mix vigorously (250 rpm on a platform shaker) at room temperature for 5 min.
15. Remove washes from spots.
    a. Add fresh 200 µL of PBS (Wash 1) to chips; or.
    b. Add fresh 200 µL of 1 M urea, 0.125% CHAPS, 0.5 M NaCl, 100 mM acetate, pH 4.5 (Wash 2) to chips.
16. Mix vigorously (250 rpm on a platform shaker) at room temperature for 5 min.
17. Repeat buffer wash one more time.
18. Wash chips 3 times with water by filling the wells and emptying.
19. Remove chips from the Bioprocessor.
20. Rinse chips with deionized water.
21. Air dry chips. Draw hydrophobic circles around spots if necessary.
22. Add 0.5 µL of SPA or CHCA to chips two times (air dry spots between additions).

C. Protein Profiling on Normal Phase ProteinChip® Array

1. Put chips in Bioprocessor. Attach the top securely.
2. Add 200 µL 50 mM HEPES pH 7.4 to each spot.
3. Mix (250 rpm on a platform shaker) at room temperature for 5 min.
4. Discard HEPES and repeat wash with fresh buffer.
5. Dilute the serum albumin depleted serum sample 10 fold in 50 mM HEPES.
6. Add 50 µL to every spot.
7. Mix (250 rpm on a platform shaker) at room temperature for 15 min.
8. Remove the samples from Normal Phase chips.
    a. Add 250 µL of HEPES (Wash 1); or
    b. Add 250 µL of 50% acetonitrile in water (Wash 2).
9. Mix (250 rpm on a platform shaker) at room temperature for 5 min.
10. Remove the washes from the spots.
    a. Add fresh 200 µL of HEPES (Wash 1); or
    b. Add fresh 200 µL of 50% acetonitrile in water (Wash 2).

11. Mix vigorously (25 °C rpm on a platform shaker) at room temperature for 5 min.
12. Remove washes.
13. Repeat wash one more time.
14. Remove chips from the Bioprocessor.
15. Air dry chips. Draw hydrophobic circles around spots if it has come off during processing.
16. Add 0.5 µL of SPA or CHCA to chips.

III. PRE-FRACTIONATION AND GAS PHASE ION SPECTROMETRY ANALYSIS, AND DETECTED MARKERS

A. Size and pI Fractionation Protocol

The basic schematic diagram of the protocol is shown in Figure 1.

1. Serum Sample Preparation

Serum samples were diluted by adjusting 20 µl of serum to 35 µl with a buffer containing 0.5 M NaCl, .1% TX-100 and Tris-HCl buffer, pH 9.0.

2. Preparation of Size Selection Columns and Size Fractionation

1. Break the outlet cap of the K30 Spin column (available from Princeton Separation, Ciphergen Biosystems, Inc., *etc.*) Insert the column into a 2 ml screw-cap tube.
2. Open the top cap of the spin column. Spin the column at 3000 rpm (720g) for 3 minutes at room temperature. The column matrix should be packed down and semi-dry, but not cracked.
3. Transfer the spin column to a new 2 m1 tube. Apply 30 µl of the diluted serum sample slowly to the center of the column matrix. Do not allow the samples to run down the side of the matrix.
4. Centrifuge columns at 3000 rpm for 3 minutes. The fractionated proteins are in the collection tube. This is fraction 1.
5. Transfer the spin column to a new tube. Add 30 µl of binding buffer (20 mM Tris-HCl, 5 mM NaCl, pH 9.0) to the column and spin as above. This is fraction 2.
6. Repeat step 5 and collect two more fractions.
7. Total of 4 fractions are collected. Store fractions on ice.

3. Preparation of Anion Exchange Column for Protein Fractionation

1. Combine fractions 1 & 2 ("Q1") and fractions 3 & 4 ("Q2") from the size exclusion spin column above. Adjust the total volume to 120 µl with binding buffer.
2. Break the outlet cap of the anion exchange Q column. Insert a 2 ml tube. Open the top cap.
3. Centrifuge the column at 1000 rpm (80g) for 1 minute. The column matrix should be packed, but not cracked.
4. Transfer the column to a new 2 ml tube. Apply 90 µl of the combined fraction samples (Q1 and Q2). Apply the sample slowly to the center of the matrix. Incubate at room temperature for 5 min.
5. Centrifuge the column(s) at 1000 rpm for 1 minute. The proteins in the collection column are the flow through fraction. These proteins do not bind to the column because they have a neutral or positive net charge in the binding buffer, or they may be coming through because the capacity of the resin is met.
6. To maximize the capture of proteins, the eluant is re-applied to the column, incubated for 3 min at room temperature, centrifuge as above. This flow through is fraction 1.
7. Transfer the column to a second tube. Wash the column with 100 µl of a buffer comprising 20 mM sodium phosphate, pH 7.0, incubate at room temperature for 3 min, centrifuge as above. Repeat this step. Collect total 200 µl for this fraction 2.
8. Transfer the column to a third tube. Apply 100 µl of a buffer comprising 50 mM sodium acetate, pH 5.0, incubate at room temperature for 3 min., centrifuge as above. This is fraction 3.
9. Transfer the column to a fourth tube. Apply 100 µl of a buffer comprising 28 mM dibasic sodium phosphate, 36 mM citrate, pH 3.4, incubate at room temperature for 3 min, centrifuge as above. This is fraction 4.
10. Transfer the column to a fifth tube. Apply 100 µl of a buffer comprising 28 mM dibasic sodium phosphate, 36 mM citrate, 1M NaCl, pH 3.4, centrifuge as above. This is fraction 5.

11. For each sample taken from a subject, 10 fractions are collected after size exclusion chromatography and the anionic exchange chromatography: Q1 Fraction 1 ("Q1F1"), Q1 Fraction 2 ("Q1F2"), Q1 Fraction 3 ("Q1F3"), Q1 Fraction 4 ("Q1F4"), Q1 Fraction 5 ("Q1F5"), Q2 Fraction 1 ("Q2F1"), Q2 Fraction 2 ("Q2F2"), Q2 Fraction 3 ("Q2F3"), Q2 Fraction 4 ("Q2F4"), Q2 Fraction 5 ("Q2F5").

4. Gas Phase Analysis of Samples

1. For all fractions except fractions Q1F5 and Q2F5, spot 2µl of each fraction on a Normal Phase ProteinChip®. Draw a hydrophobic ring around each array before spotting. Let it dry completely before spotting 0.5µl SPA matrix twice using 50% acetonitrile with .5% TFA, and 0.6 % Triton X as the solvent. If desired, CHCA can be used as a matrix material. For Q1F5 and Q2F5 that have a high salt content, spot 2µl of each fraction on a H4 Chip. After incubating for about 5 minutes at room temperature, wash the spots with water. Let it dry completely before adding a matrix material.
2. Analyze the probe using a mass spectrometer (*e.g.*, ProteinChip® System). Read the chips on laser intensity 15, sensitivity of 10, with the filter in for a low mass reading and at laser intensity 50 with the filter in for high mass collection. Collect 70 shots for the combined average spectra (10 shots over 7 regions, *i.e.*, 20-80).
3. View the spectra for each reading and use a filter (50 and 150 is adequate for L15 and L50, respectively). Use the auto-peak identification mode with settings of 5 times the noise.

5. Markers Detected

18 serum samples from breast cancer patients (Stage III & IV) and 18 serum samples from control women (*i.e.*, women in whom breast cancer was undetectable) were prepared as described above. Ten fractions from each person were separately analyzed. All fractions except fractions Q1F5 and Q2F5 were analyzed using the Normal Phase ProteinChip®. Since Q1F5 and Q2F5 fractions have high salt content, these fractions were analyzed using the H4 ProteinChip®.

The following markers were detected from the above samples: Marker BrH1; Marker BrH3; Marker BrH5; Marker BrH4; Marker BrH2; Marker BrH7; and Marker BrH6. Marker BrH1 was mainly eluted and detected in Q1F1 fraction (pH 9 fraction) and was also detected in Q1F2 fraction (pH 7 fraction). Marker BrH2 was mainly eluted and detected in Q2F1 fraction (pH 9 fraction) and was also eluted and detected in Q1F2 fraction (pH 7 fraction). Marker BrH3 was mainly eluted and detected in Q1F1 fraction (pH 9 fraction) and was also eluted and detected in Q2F1 (pH 9 fraction). Marker BrH4 was mainly eluted and detected in Q2F4 fraction (pH 3.4 fraction) and was also eluted and detected in Q1F4 fraction (pH 3.4 fraction). Marker BrH5 was mainly detected in Q1F1 fraction (pH 9 fraction). Marker BrH6 was mainly eluted and detected in Q1F4 fraction (pH 3.4). Marker BrH7 was mainly eluted and detected in Q1F4 (pH 3.4 fraction).

Figure 8A illustrates the analysis of spectral view data using Spotfire Pro analysis database (Spotfire, Inc., Cambridge, MA). The left block shows the spectral views of detected proteins from 18 breast cancer samples. The right block shows the spectral view of detected proteins from 18 control samples. The middle block shows the molecular weight and intensity of each protein shown in the spectral views using Spotfire Pro analysis database. The detected proteins from breast cancer samples are shown as black circles, and the detected proteins from control samples are shown as gray circles. The average intensity of the peak from marker BrH5 (15220 ± 30 Da) is higher in breast cancer samples than in control samples, indicating that this marker is up-regulated in many breast cancer patients' serum. Figures 8B and 8C illustrate database analysis by Spotfire Pro software for markers BrH1 and BrH3.

The summary of all the markers detected using the size and pI fractionation protocol is shown in Figure 7. As shown in the table in Figure 7, for each sample (18 breast cancer samples and 18 control samples), the detection of a marker is noted as "X". For each marker, the frequency of the markers observed in breast cancer patients' samples compared to in control samples as shown under column "B/C." The signal intensity of markers observed in breast cancer patients' samples compared to that observed in control samples is also shown. Out of seven markers, markers BrH1, BrH3, BrH5 and BrH6 were detected at both higher levels and higher frequency in the breast cancer patients' serum samples than in the control serum samples.

B. THE STANDARD PROTOCOL, MODIFIED STANDARD PROTOCOL, pH GRADIENT PROTOCOL, AND MARKERS DETECTED

1. Protocols and Sample Preparation

A "standard protocol" refers to a pre-fractionation protocol which comprises applying a sample to a Cibacron blue column to remove serum albumin present in the sample. A "modified standard protocol" refers to a pre-fractionation protocol which comprises applying a sample to an anti-human serum albumin column to remove serum albumin present in the sample. Schematic diagrams of "a standard protocol" and "a modified standard protocol" are shown in Figures 2 and 3, respectively.

Buffers:

Prepare a buffer of 8 M urea, 1% CHAPS, 1x PBS.

Prepare a second buffer of 1 M urea, 0.125% CHAPS, 1x PBS.

Method:

1. To 40 µL human serum add 60 µL 8M urea (*e.g.*, Sigma electrophoresis grade), 1% CHAPS in PBS.
2. Vortex in cold, 10 min.
3. Prepare Cibacron Blue spin column. Alternatively, prepare anti-HSA antibody coupled beads. Cibacron Blue agarose and anti-HSA can be purchased from Sigma. Sheep anti-human serum albumin antibody was also obtained from Biodesign, Saco, Maine. The antibodies can be covalently coupled to preactivated beads (*e.g.*, from Pierce) and packed into column. Start with 150 µL of a 50% (v/v) bead suspension. Equilibrate with 3 x 300 µL 1M urea, 0.125% CHAPS, PBS. Spin column dry at 1000 x g, 30 sec each time.
4. Add 100 µL diluted serum sample to column.
5. Wash original serum tube with 100 µL 1M urea, 0.125% CHAPS, PBS. Add wash to column.
6. Put column into fresh collection tube. Vortex in cold, 15 min.
7. Centrifuge column at 1000 x g, 30 sec. Save filtrate in the collection tube.
8. Add 100 µL 1 M urea, 0.125% CHAPS, PBS to the column.
   Note the decreased amount of urea and CHAPS in this buffer.
9. Put column into fresh collection tube. Vortex in cold, 15 min.

10. Put column back into first collection tube. Spin column 1000 x g, 30 sec.

11. Pool the 2 filtrates. Volume should equal approximately 300 µL.

12. Place about 2 µL of a sample on a probe, add a matrix material and perform gas phase ion spectrometry analysis as described above.

2. Markers Detected

18 serum samples from breast cancer patients (Stage III & IV) and 18 serum samples from control women (*i.e.*, women with undetectable breast cancer) were prepared as described above. The prepared samples were analyzed using the IMAC3-Cu^{++} chip.

The following markers were detected from the above samples: BrM1; BrM2; BrM3; BrM4; and BrM5. The majority of these markers were detected regardless of whether Cibacron Blue column or an anti-HSA column was used to remove serum albumin. Some of the markers were better resolved using Wash 1 (50 mM HEPES), while other markers were better resolved using Wash 2 (1M urea, 0.125% CHAPS, 0.25 M NaCl, 50 mM HEPES). Certain markers were better resolved using SPA, while other markers were better resolved using CHCA as an energy absorbing material.

Figures 9-11 illustrate data obtained using the standard protocol (*i.e.*, removing serum albumin using a Cibacron Blue column and detecting the protein using the Cu(II) chip). Figure 9A illustrates a Spotfire Scatter Plot of marker BrM1 detected in 18 breast cancer patients' samples and 18 control samples. Figure 9B illustrates a Spotfire Scatter Plot of marker BrM2 in 18 breast cancer patients' samples and 18 control samples. Figure 10A illustrates a Spotfire Scatter Plot of marker BrM3. Figure 10B illustrates a gel view of marker BrM3 in the control samples and in the breast cancer patients' samples. Figure 11A illustrates a Spotfire Scatter Plot of marker BrM4 detection. Figure 11B illustrates a gel view of marker BrM4 detection when the probe was washed with wash 1 solution, and Figure 11C illustrates a gel view of marker BrM4 when the probe was washed with wash 2 solution. As seen in the two figures, the resolution of markers is different depending on the wash solution used.

C. THE ION EXCHANGE SPIN COLUMN PROTOCOL AND MARKERS DETECTED

1. Protocol and Sample Preparation

The schematic diagram of the ion exchange spin column protocol is shown in Figure 4.

1. To 20 μL human serum, add 30 μL 8M urea (Sigma electrophoresis grade), 1% CHAPS (Sigma), PBS.
2. Prepare Q-HF spin column (Q Hyper D HF obtained from Biosepra, Life Technologies Inc., Rockville, MD) by equilibrating 125 μL 50% (v/v) Biosepra bead suspension with 3x 300 μL of 0.1 M sodium bicarbonate, pH 8.2. Spin column dry at 1000 xg 30 seconds each time.
3. Add 50 μL diluted serum sample to column. Wash original serum tube with 50 μL of 0.1 M sodium bicarbonate, pH 8.2. Add wash to column.
4. Vortex 5 seconds. Stand in cold for 2 minutes.
5. Spin column 1000 xg 30 seconds. Save filtrate in the collection tube (Q1 fraction).
6. Add 100 μL of 0.1 M ammonium acetate, pH 7.0 to the column.
7. Vortex for 5 seconds. Stand in cold for 2 minutes.
8. Spin column 1000 xg 30 seconds. Pool into the Q1 fraction.
9. Add 100 μL 0.1 M sodium acetate, pH 5.0 to the column.
10. Put column into a fresh collection tube. Vortex for 5 seconds. Stand in cold for 2 minutes.
11. Spin column 1000 xg 30 seconds. Save filtrate in the collection tube (Q2 fraction).
12. Add 100 μL 0.1 M sodium citrate, pH 3.0 to the column.
13. Put column into a fresh collection tube. Vortex in for 5 seconds. Stand in the cold for 2 minutes.
14. Spin column 1000 xg 30 seconds. Save filtrate in the collection tube (Q3 fraction).
15. Add 100 μL 50% isopropanol: acetonitrile (2:1) 0.1% TFA to the column.
16. Put column into a fresh collection tube. Vortex in for 5 seconds. Stand in the cold for 2 minutes.
17. Spin column 1000 xg 30 seconds. Save filtrate in the collection tube (Q4 fraction).
18. Place about 2 μL of a sample on a probe, add a matrix material and perform gas phase ion spectrometry analysis as described above.

2. Markers Detected

18 serum samples from breast cancer patients (Stage III & IV) and 18 serum samples from control women (*i.e.*, women with undetectable breast cancer) were prepared as described above. The prepared samples were analyzed using the IMAC3-Cu^{++} chip.

The marker having the following apparent molecular weight was detected from the above samples: BrM4. This marker was detected in Q3 fraction using a IMAC3-Cu^{++} chip using SPA as the energy absorbing material.

D. THE TANDEM SEQUENCE EXTRACTION PROTOCOL AND MARKERS DETECTED

1. Protocol and Sample Preparation.

The schematic diagram of a tandem sequence extraction protocol is shown in Figure 5. While the tandem sequence extraction protocol in Figure 5 uses the SAX2 chip, the Wheat Germ Lectin chip, the IMAC3-Cu^{++} chip, and the Heparin chip, a number of other chips with different surface chemistries can be used to extract different proteins. Illustrated below are the tandem sequence extraction protocol that is shown on the leftmost column of the schematic diagram of Figure 5 (*i.e.*, tandem sequence extraction in the order of SAX2 chip, Wheat germ lectin chip, Cu(II) chip, Heparin chip). However, as shown in the diagram, other permutations of tandem sequence extraction protocols may be used to extract different markers at each level.

a) Protein Profiling on SAX2 Chip

S1. Put SAX2 chips into Bioprocessor. After the top is in place, add 100 µL 50 mM HEPES buffer pH 7.0 to all spots.

S2. Mix (250 rpm on a platform shaker) at room temperature for 5 minutes.

S3. Discard buffer and repeat wash with fresh buffer.

S4. Dilute the serum sample 1:6 in 50 mM HEPES buffer, pH 7.0. Add 50 µL to appropriate spot. Seal the Bioprocessor with tape.

S5. Mix (250 rpm on a platform shaker) at room temperature for 30 min.

S6. Remove the samples from SAX2 chip. (Transfer samples to Lectin chip).

S7. Add 50 µL of 50 mM HEPES buffer, pH 7.0 to all spots.

S8. Mix (250 rpm on a platform shaker) at room temperature for 30 min.

S9. Remove the washes from the spots. (Transfer washes to Lectin chip). Add fresh 50 µL of 50 mM HEPES buffer, pH 7.0 to all spots.

S10. Mix (250 rpm on a platform shaker) at room temperature for 15 mm.

S11. Remove the washes from the spots. (Transfer washes to Lectin chip).

S12. Wash chip 3 times with water by filling the wells and emptying. Remove chip from Bioprocessor.

S13. Rinse chip with running water.

S14. Air dry chip.

S15. Add 0.5 μL of SPA to all spots two times (air dry spots between additions).

b) Protein Profiling on Wheat Germ Lectin Chip

L1. Put Wheat Germ Lectin chips into Bioprocessor. After the top is in place, add 100 μL 50 mM HEPES buffer pH 7.0 to all spots.

L2. Mix (250 rpm on a platform shaker) at room temperature for 5 min.

L3. Discard buffer and repeat wash with fresh buffer.

L4. Transfer samples from SAX2 chip to corresponding Lectin chip spots. Seal the Bioprocessor with tape.

L5. Mix (250 rpm on a platform shaker) at room temperature for 30 min.

L6. Remove the samples from Lectin chip. (Transfer samples to Cu(II) chip).

L7. Transfer washes from SAX2 chip to corresponding Lectin chip spots.

L8. Mix (250 rpm on a platform shaker) at room temperature for 15 min.

L9. Remove the washes from the spots. (Transfer washes to Cu(II) chip). Transfer second washes from SAX2 chip to corresponding Lectin chip spots.

L10. Mix (250 rpm on a platform shaker) at room temperature for 15 min.

L11. Remove the washes from the spots. (Transfer washes to Cu(II) chip).

L12. Wash chip 3 times with water by filling the wells and emptying. Remove chip from Bioprocessor.

L13. Rinse chip with running water.

L14. Air dry chip.

L15. Add 0.5 μL of SPA to all spots two times (air dry spots between additions).

c) Protein Profiling on IMAC3-Cu^{++} Chip

C1. Add 5 μL of 50 mM Cu(II) to each spot of IMAC3 chip.

C2. Mix at room temperature for 5 min.

C3. Rinse chip with water.

C4. Wash chip with 100 mM sodium acetate pH 4.0 two times. Each time mix at room temperature for 5 min.

C5. Put Cu(II) chip into Bioprocessor. After the top is in place, add 100 µL 50 mM HEPES buffer pH 7.0 to all spots.

C6. Mix (250 rpm on a platform shaker) at room temperature for 5 min.

C7. Discard buffers and repeat wash with fresh buffers.

C8. Transfer samples from Lectin chip to corresponding Cu(II) chip spots. Seal the Bioprocessor with tape.

C9. Mix (250 rpm on a platform shaker) at room temperature for 15 min.

C10. Remove the samples from Cu(II) chip.

C11. Transfer washes from Lectin chip to corresponding Cu(II) chip spots.

C12. Mix (250 rpm on a platform shaker) at room temperature for 15 min.

C13. Remove the washes from Cu(II) chip.

C14. Transfer second washes from Lectin chip to corresponding Cu(II) chip spots.

C15. Mix (250 rpm on a platform shaker) at room temperature for 15 min.

C16. Remove the washes from the spots.

C17. Wash chip 3 times with water by filling the wells and emptying. Remove chips from Bioprocessor.

C18. Rinse chip with running water.

C19. Air dry chip.

C20. Add 0.5 µL of SPA to all spots two times (air dry spots between additions).

2. Markers Detected

Using the tandem extraction protocol described above, the following markers were detected: Marker BrM2 on the Lectin chip; and Marker BrM5 on the Cu(II) chip using the SPA.

The present invention provides novel materials and methods for aiding breast cancer diagnosis using markers that are differentially present in samples of a breast cancer patient and a normal subject who does not have breast cancer. While specific examples have been provided, the above description is illustrative and not restrictive.

Any one or more of the features of the previously described embodiments can be combined in any manner with one or more features of any other embodiments in the present invention. Furthermore, many variations of the invention will become apparent to those skilled in the art upon review of the specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted. By their citation of various references in this document, Applicants do not admit any particular reference is "prior art" to their invention.

WHAT IS CLAIMED IS:

1. A method for aiding a breast cancer diagnosis, the method comprising:

(a) detecting at least one protein marker in a sample, wherein the protein marker is selected from:

Marker BrH1: 6850 ± 14 Da;

Marker BrH2: 8565 ± 17 Da;

Marker BrH3: 8920 ± 18 Da;

Marker BrH4: 11180 ± 22 Da;

Marker BrH5: 15220 ± 30 Da;

Marker BrH6: 106080 ± 530 Da;

Marker BrH7: 117600 ± 590 Da;

Marker BrM1: 2804 ± 6 Da;

Marker BrM2: 3390 ± 7 Da;

Marker BrM3: 5890 ± 12 Da;

Marker BrM4: 11900 ± 24 Da; and

Marker BrM5: 51000 ± 260 Da; and

(b) correlating the detection of the marker or markers with a probable diagnosis of breast cancer.

2. The method of claim 1, wherein the correlation takes into account the amount of the marker or markers in the sample compared to a control amount of the marker or markers.

3. The method of claim 1, wherein the correlation takes into account the presence or absence of the marker or markers in the sample and the frequency of detection of the same marker or markers in a control.

4. The method of claim 2, wherein the correlation further takes into account the presence or absence of the marker or markers in the sample and the frequency of detection of the same marker or markers in a control.

5. The method of claim 1, wherein the method comprises detecting a plurality of the markers.

6. The method of claim 1, wherein the method comprises detecting at least three of the markers.

7. The method of claim 1, wherein the method comprises detecting at least four of the markers.

8. The method of claim 1, wherein the method comprises detecting at least five of the markers.

9. The method of claim 1, wherein detecting the presence of at least four of five markers BrH1, BrH3, BrH5, BrH6 and BrH7 is highly correlated with a positive diagnosis of breast cancer.

10. The method of claim 1, wherein detecting the absence of four of five markers BrH1, BrH3, BrH5, BrH6 and BrH7 is highly correlated with a negative diagnosis of breast cancer.

11. The method of claim 1, wherein the sample is blood serum.

12. The method of claim 1, wherein gas phase ion spectrometry is used for detecting the marker or markers.

13. The method of claim 12, wherein the gas phase ion spectrometry is laser desorption/ionization mass spectrometry.

14. The method of claim 13, wherein laser desorption/ionization mass spectrometry comprises:

(a) providing a substrate comprising an adsorbent attached thereto;

(b) contacting the sample with the adsorbent; and

(c) desorbing and ionizing the marker or markers from the substrate and detecting the desorbed/ionized marker or markers with the mass spectrometer.

15. The method of claim 14, wherein the substrate is a probe adapted for use with the mass spectrometer.

16. The method of claim 14, wherein the substrate is suitable for being placed on a probe which is adapted for use with the mass spectrometer.

17. The method of claim 14, wherein the adsorbent is an antibody that specifically binds to the marker.

18. The method of claim 14, wherein the adsorbent is a hydrophilic adsorbent.

19. The method of claim 18, wherein the hydrophilic adsorbent is silicon oxide.

20. The method of claim 18, comprising detecting one or more of markers BrH1, BrH2, BrH3, BrH4, BrH5, BrH6, and BrH7.

21. The method of claim 14, wherein the adsorbent is a metal chelating adsorbent.

22. The method of claim 21, comprising detecting one or more of markers BrM1, BrM2, BrM3, BrM4, and BrM5.

23. The method of claim 14, wherein the adsorbent is a lectin adsorbent.

24. The method of claim 23, comprising detecting marker BrM2.

25. The method of claim 1, wherein an immunoassay is used for detecting the marker or markers.

26. The method of claim 13, the method further comprising:

(a) generating data on the sample with the mass spectrometer indicating intensity of signal for mass/charge ratios;

(b) transforming the data into computer-readable form; and

(c) operating a computer to execute an algorithm, wherein the algorithm determines closeness-of-fit between the computer-readable data and data indicating a diagnosis of breast cancer or a negative diagnosis.

27. A method for detecting at least one protein marker in a sample, wherein the marker is selected from:

Marker BrH1: 6850 ± 14 Da;

Marker BrH2: 8565 ± 17 Da;

Marker BrH3: 8920 ± 18 Da;

Marker BrH4: 11180 ± 22 Da;

Marker BrH5: 15220 ± 30 Da;

Marker BrH6: 106080 ± 530 Da;

Marker BrH7: 117600 ± 590 Da;

Marker BrM1: 2804 ± 6 Da;

Marker BrM2: 3390 ± 7 Da;

Marker BrM3: 5890 ± 12 Da;

Marker BrM4: 11900 ± 24 Da; and

Marker BrM5: 51000 ± 260 Da;

wherein the method comprises detecting the marker or markers by gas phase ion spectrometry.

28. The method of claim 27, wherein the sample is a blood serum sample.

29. The method of claim 27, wherein the detection method comprises detecting the marker or markers by laser desorption/ionization mass spectrometry.

30. The method of claim 27, further comprising comparing the amount of the detected marker or markers to a control.

31. The method of claim 29 comprising:

(a) generating data on the sample with the mass spectrometer indicating intensity of signal for mass/charge ratio;

(b) transforming the data into computer-readable form; and

(c) operating a computer and executing an algorithm that detects signal in the computer-readable data representing the marker or markers.

32. The method of claim 29, wherein laser desorption/ionization mass spectrometry comprises:

(a) providing a substrate comprising an adsorbent attached thereto;

(b) contacting the sample with the adsorbent; and

(c) desorbing and ionizing the marker or markers from the substrate and detecting the desorbed/ionized marker or markers with the mass spectrometer.

33. The method of claim 32, wherein the substrate is a probe adapted for use with the mass spectrometer.

34. The method of claim 32, wherein the substrate is suitable for being placed on a probe which is adapted for use with the mass spectrometer.

35. The method of claim 29 comprising:

(a) fractionating the sample by size exclusion chromatography and by anion exchange chromatography, and collecting a sample fraction that includes the marker or markers;

(b) contacting the sample fraction with an adsorbent on a substrate;

(c) desorbing and ionizing the marker or markers retained on the adsorbent and detecting the desorbed/ionized marker or markers with the mass spectrometer.

36. The method of claim 35, wherein the adsorbent is a hydrophilic adsorbent.

37. The method of claim 36, wherein the hydrophilic adsorbent comprises silicon oxide.

38. The method of claim 35, comprising detecting one or more of markers BrH1, BrH2, BrH3, BrH4, BrH5, BrH6, and BrH7.

39. The method of claim 29 comprising:

(a) removing serum album from the sample and collecting a sample fraction that includes the marker or markers;

(b) contacting the sample fraction with an adsorbent on a substrate;

(c) desorbing and ionizing marker or markers retained on the adsorbent and detecting the desorbed/ionized marker or markers with the mass spectrometer.

40. The method of claim 39, wherein the adsorbent is an antibody that specifically binds one of more of the markers.

41. The method of claim 39, wherein the adsorbent is a metal chelate adsorbent.

42. The method of claim 41, comprising detecting one or more of markers BrM1, BrM2, BrM3, BrM4, and BrM5.

43. The method of claim 29, the method comprising:

(a) fractionating the sample by anion exchange chromatography and collecting a sample fraction that includes the marker or markers;

(b) contacting the sample fraction with an adsorbent on a substrate;

(c) desorbing and ionizing marker or markers retained on the adsorbent and detecting the desorbed/ionized marker or markers with the mass spectrometer.

44. The method of claim 43, wherein the adsorbent is a metal chelate adsorbent.

45. The method of claim 44, wherein the detected marker is BrM5.

46. The method of claim 29, the method comprising:

(a) removing serum albumin from the sample, and collecting a first sample fraction that includes the marker or markers;

(b) contacting the first sample fraction with a cationic adsorbent and collecting a second fraction that is not retained by the cationic adsorbent;

(c) contacting the second sample fraction with a lectin adsorbent;

(d) desorbing and ionizing marker or markers retained on the lectin adsorbent and detecting the desorbed/ionized marker or markers with the mass spectrometer.

47. The method of claim 46, comprising detecting marker BrM2.

48. The method of claim 29 comprising:

(a) removing serum albumin from the sample, and collecting a first sample fraction that includes the marker or markers;

(b) contacting the first sample fraction with a cationic adsorbent and collecting a second sample fraction that is not retained by the cationic adsorbent;

(c) contacting the second sample fraction with a lectin adsorbent and collecting a third sample fraction that is not retained by the lectin adsorbent;

(d) contacting the third sample fraction on a metal chelate adsorbent;

(d) desorbing and ionizing marker or markers retained on the metal chelate adsorbent and detecting the desorbed/ionized marker or markers with the mass spectrometer.

49. The method of claim 48, comprising detecting marker BrM5.

50. The method of claim 27, wherein the method further comprises, prior to detection,

(a) separating biomolecules in the sample into one or two-dimensional array of spots comprising one or more biomolecules;

(b) selecting and removing a spot from the array which is suspected of comprising the marker or markers; and

(c) analyzing the selected spot by gas phase ion spectrometry to determine if the selected spot comprises the marker or markers.

51. The method of claim 50 further comprising comparing the amount of the detected marker or markers with a control.

52. The method of claim 50, wherein the method further comprises digesting the biomolecules in the selected spot by an enzyme prior to analyzing the selected spot by gas phase ion spectrometry.

53. The method of claim 27, wherein the method further comprises, prior to detection,

(a) separating biomolecules in the sample by high performance liquid gas chromatography;

(b) collecting a fraction suspected of comprising the marker or markers; and

(c) analyzing the fraction by gas phase ion spectrometry to determine if the fraction comprises the marker or markers.

54. The method of claim 53 further comprising comparing the amount of the detected marker or markers with a control.

55. A method for detecting at least one protein marker in a sample, wherein the marker is selected from:

Marker BrH1: 6850 ± 14 Da;

Marker BrH2: 8565 ± 17 Da;

Marker BrH3: 8920 ± 18 Da;

Marker BrH4: 11180 ± 22 Da;

Marker BrH5: 15220 ± 30 Da;

Marker BrH6: 106080 ± 530 Da;

Marker BrH7: 117600 ± 590 Da;

Marker BrM1: 2804 ± 6 Da;

Marker BrM2: 3390 ± 7 Da;

Marker BrM3: 5890 ± 12 Da;

Marker BrM4: 11900 ± 24 Da; and

Marker BrM5: 51000 ± 260 Da;

wherein the method comprises detecting the marker or markers by an immunoassay.

56. A purified protein selected from:

Marker BrH1: 6850 ± 14 Da;

Marker BrH2: 8565 ± 17 Da;

Marker BrH3: 8920 ± 18 Da;

Marker BrH4: 11180 ± 22 Da;

Marker BrH5: 15220 ± 30 Da;

Marker BrH6: 106080 ± 530 Da;

Marker BrH7: 117600 ± 590 Da;

Marker BrM1: 2804 ± 6 Da;

Marker BrM2: 3390 ± 7 Da;

Marker BrM3: 5890 ± 12 Da;

Marker BrM4: 11900 ± 24 Da; and

Marker BrM5: 51000 ± 260 Da.

57. A kit comprising:

(a) a substrate comprising an adsorbent attached thereto, wherein the adsorbent is capable of retaining at least one protein marker selected from:

Marker BrH1: 6850 ± 14 Da;

Marker BrH2: 8565 ± 17 Da;

Marker BrH3: 8920 ± 18 Da;

Marker BrH4: 11180 ± 22 Da;

Marker BrH5: 15220 ± 30 Da;

Marker BrH6: 106080 ± 530 Da;

Marker BrH7: 117600 ± 590 Da;

Marker BrM1: 2804 ± 6 Da;

Marker BrM2: 3390 ± 7 Da;

Marker BrM3: 5890 ± 12 Da;

Marker BrM4: 11900 ± 24 Da; and

Marker BrM5: 51000 ± 260 Da; and

(b) instructions to detect the marker or markers by contacting a sample with the adsorbent and detecting the marker or markers retained by the adsorbent.

58. The kit of claim 57, wherein the substrate is a probe adapted for use with a gas phase ion spectrometer, the probe having a surface onto which the adsorbent is attached.

59. The kit of claim 57, wherein the substrate is suitable for being placed on a probe adapted for use with a gas phase ion spectrometer.

60. The kit of claim 59, the kit further comprising the probe adapted for use with a gas phase ion spectrometer.

61. The kit of claim 57, wherein the adsorbent is a metal chelate adsorbent.

62. The kit of claim 57, wherein the adsorbent comprises a hydrophilic group.

63. The kit of claim 62, wherein the hydrophilic adsorbent comprises silicon oxide.

64. The kit of claim 57, wherein the adsorbent comprises lectin.

65. The kit of claim 57, wherein the adsorbent comprises a cationic group.

66. The kit of claim 57, wherein the adsorbent is an antibody that specifically binds to the marker or markers.

67. The kit of claim 57, wherein the kit further comprises a reference.

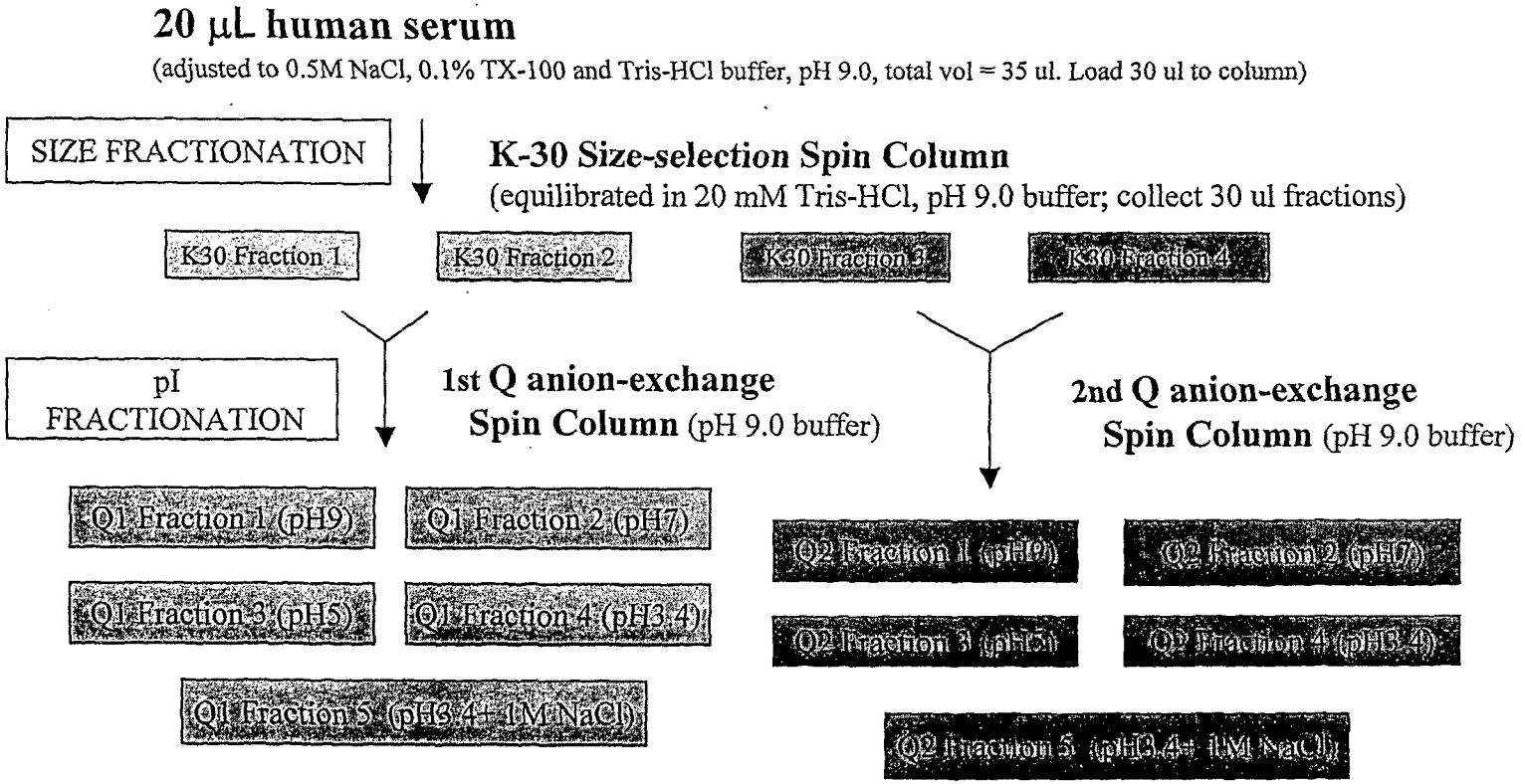
68. The kit of claim 67, wherein the reference is an antibody that binds to a serum protein.

69. The kit of claim 57, wherein the substrate comprises a plurality of different types of adsorbents.

70. The method of claim 57, the kit further comprising (1) an eluant wherein the marker or markers are retained on the adsorbent when washed with the eluant, or (2) instructions to wash adsorbent with the eluant after contacting the adsorbent with marker or markers.

SELDI-Assisted™ Protein Fractionation

Fractionation and enrichment of proteins from Serum



(Each Q column fraction is 150 ul)

FIG 1

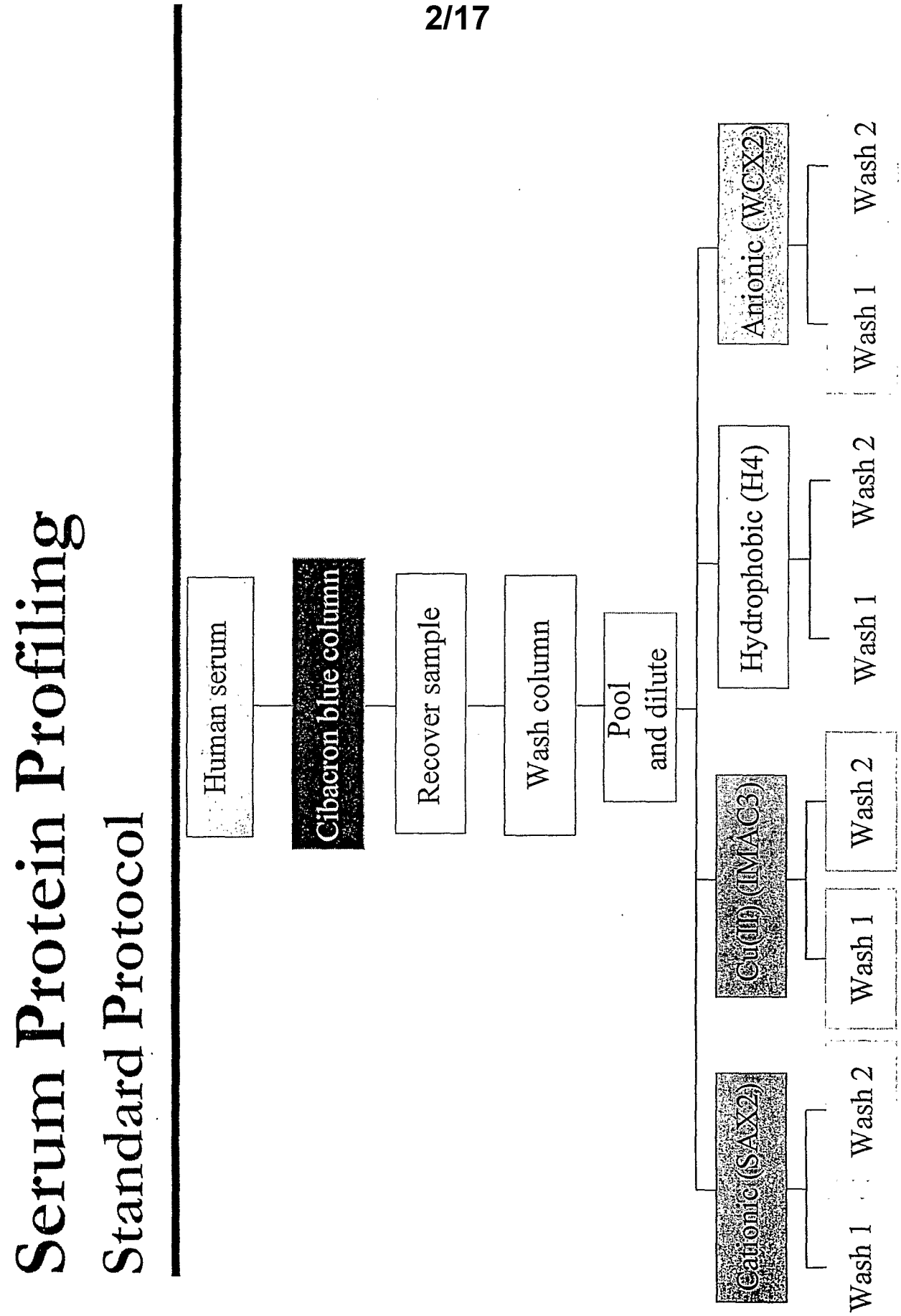

Serum Protein Profiling
Standard Protocol
Human serum
Cibacron blue column
Recover sample
Wash column
Pool and dilute
Cationic (SAX2)
Cu(II) (IMAC3)
Hydrophobic (H4)
Anionic (WCX2)
Wash 1
Wash 2
Wash 1
Wash 2
Wash 1
Wash 2
Wash 1
Wash 2

FIG 2

Serum Protein Profiling

Modified Standard Protocol

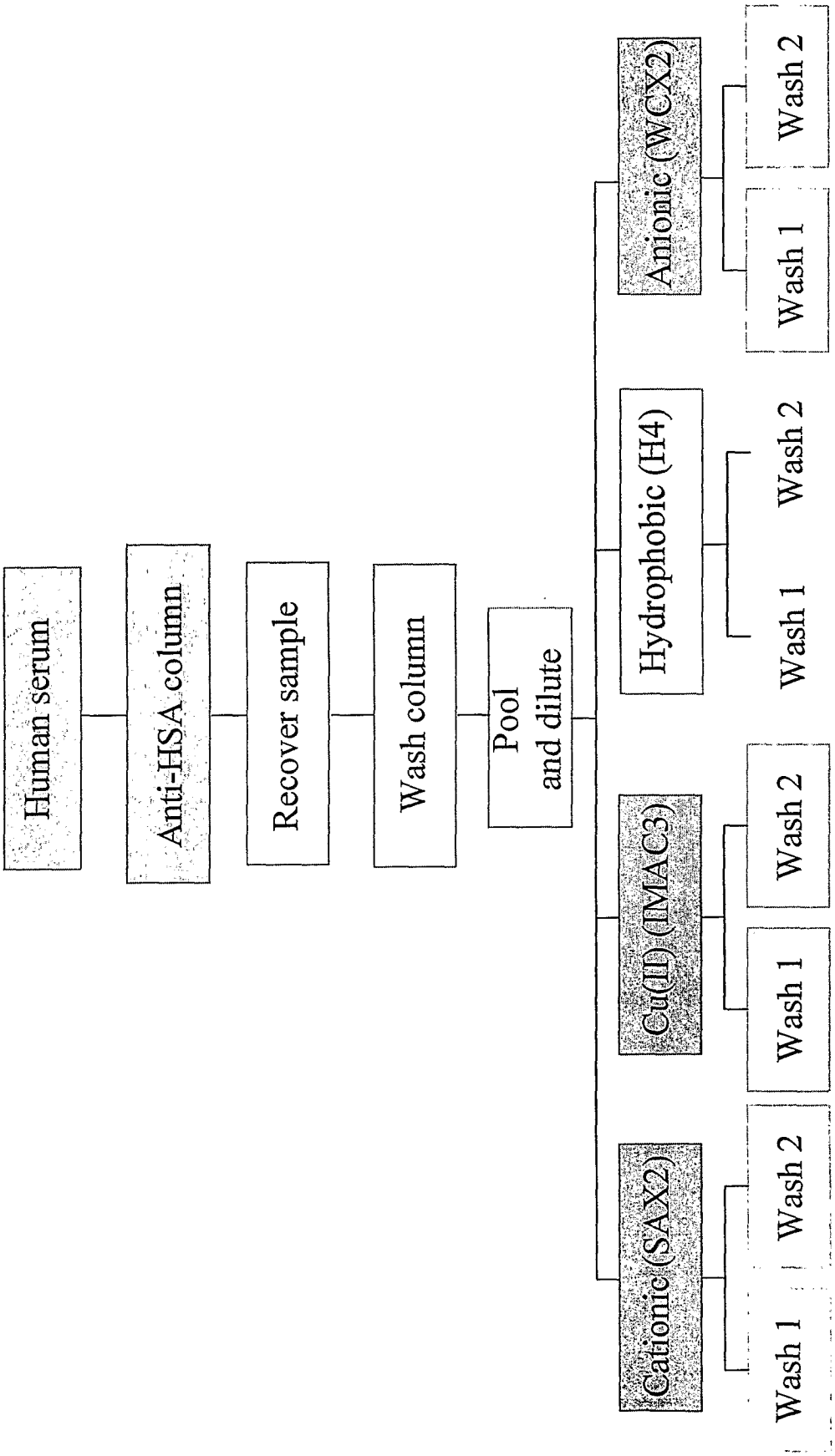


FIG 3

Serum Protein Profiling
Ion Exchange Spin Column Protocol

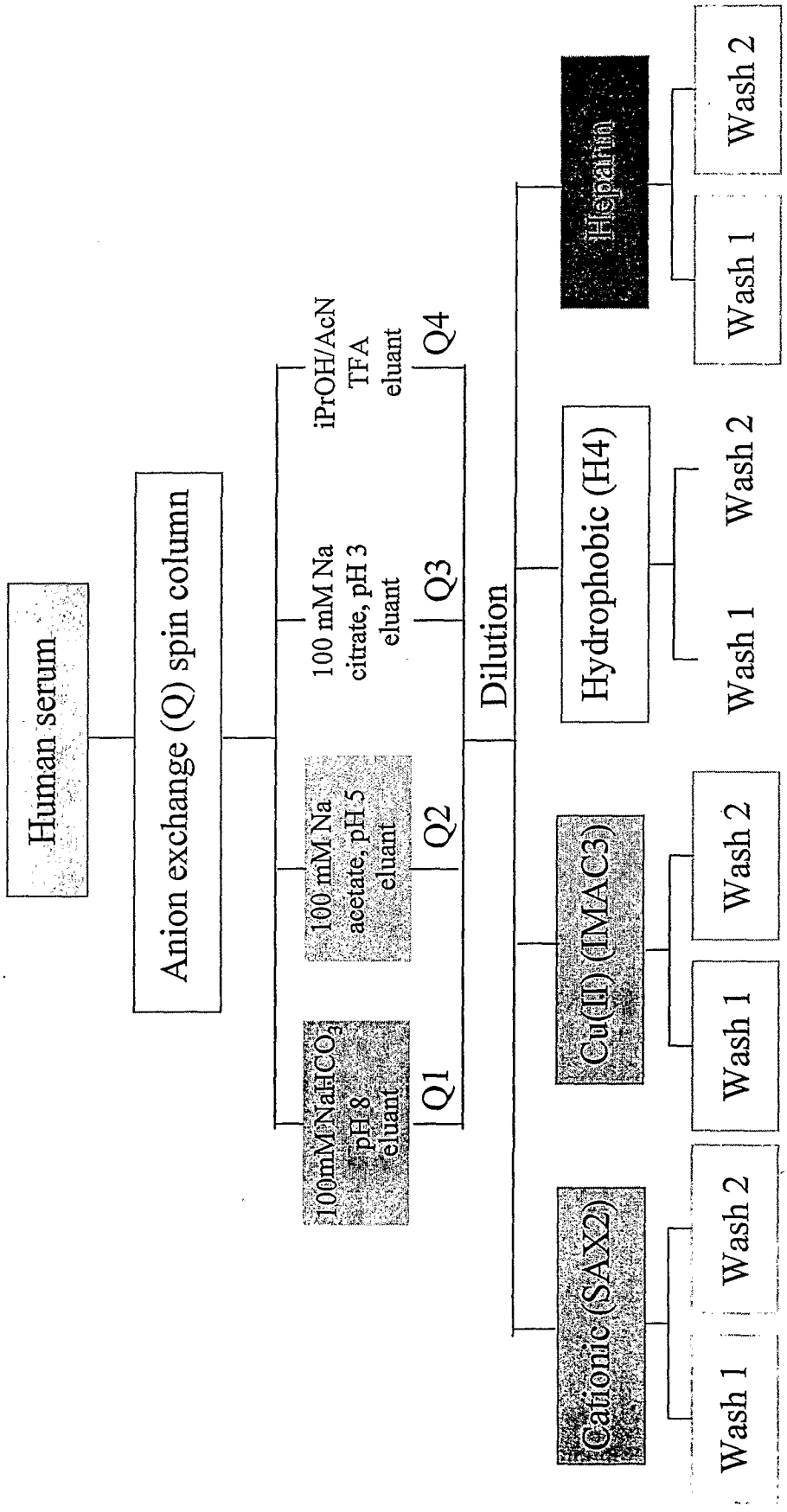


FIG 4

Serum Protein Profiling Tandem Sequence Extraction Protocol

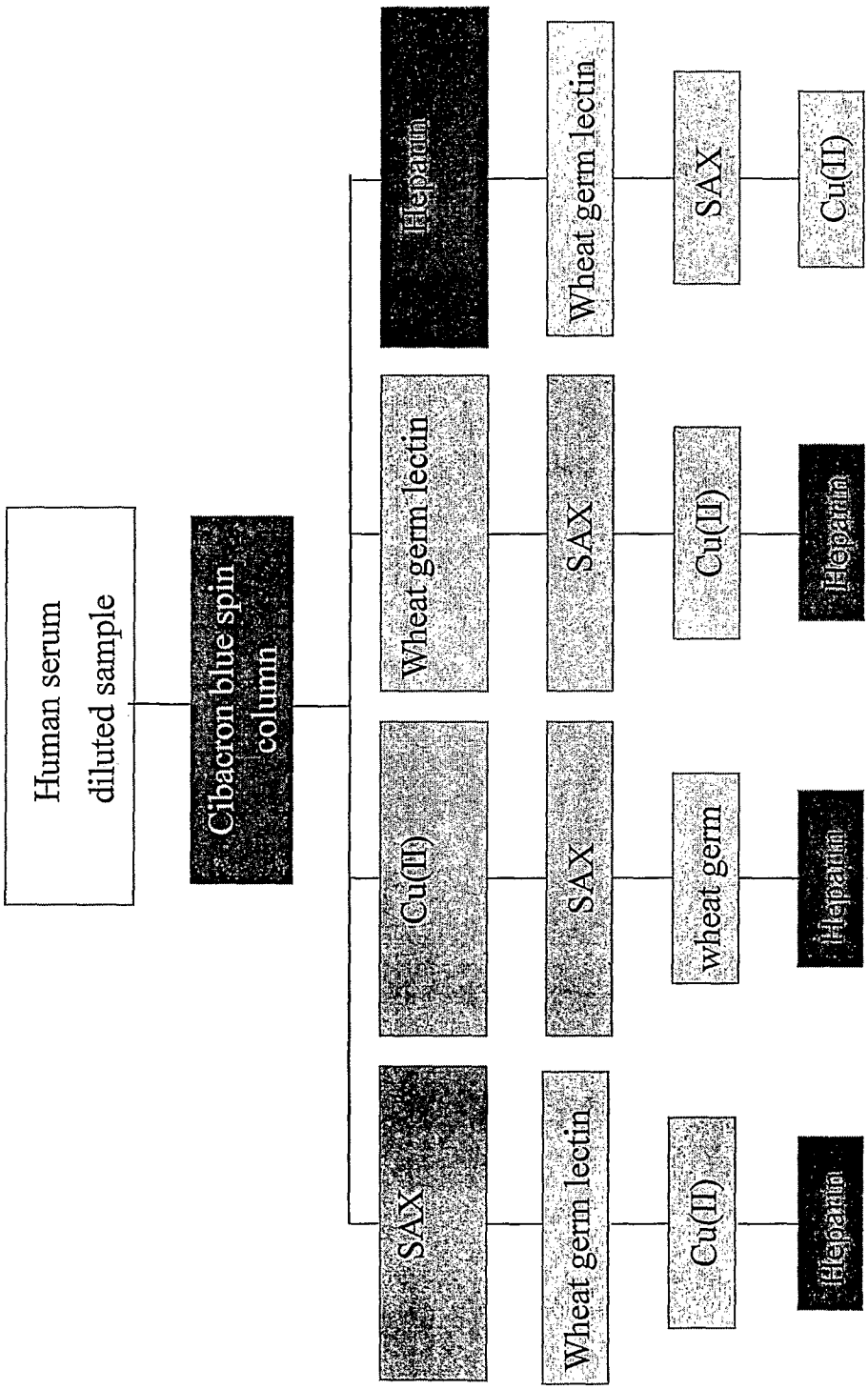


FIG 5

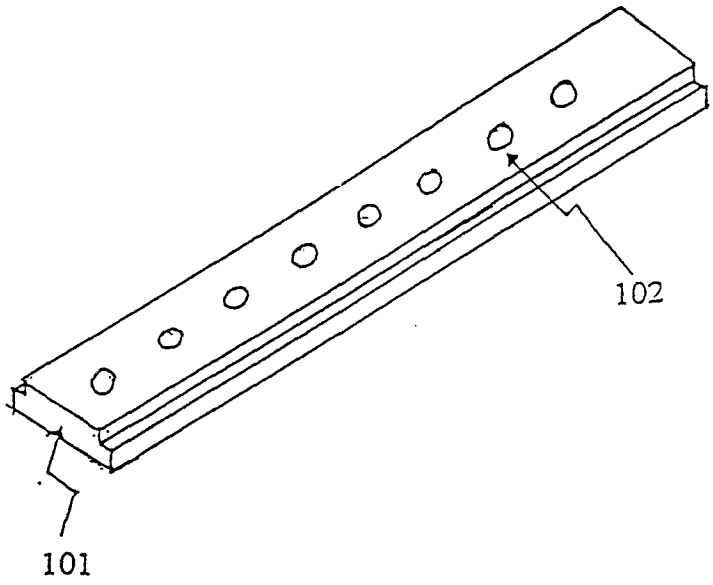


FIG 6

Potential Protein markers in Breast cancer serum

Breast Cancer Serum Biomarkers

MARKERS		SAMPLES																		FREQUENCY		SIGNAL INTENSITY			
Avg. MW		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	Total	B/C	Med	B/C	Avg	B/C
6848.81	B	X	X		X	X		X	X	X	X	X	X			X		X	X	13	1.86	0.70	3.40	0.78	1.35
	C						X		X	X		X				X	X	X		7		0.21		0.58	
8919.21	B		X	X			X	X			X	X	X	X	X	X	X	X		12	2.00	0.42	5.33	0.88	9.84
	C		X									X	X		X	X			X	6		0.08		0.09	
15223.82	B	X	X	X	X	X		X	X	X					X			X	X	11	1.83	0.68	1.28	0.71	1.32
	C	X						X			X	X		X			X			6		0.53		0.54	
11183.24	B			X	X		X					X	X	X	X		X	X	X	10	1.00	0.62	2.77	0.76	2.15
	C		X		X	X		X	X			X	X		X	X		X		10		0.22		0.35	
8563.79	B			X							X	X	X	X	X	X	X	X	X	10	1.11	0.66	2.17	0.90	2.60
	C										X	X	X	X	X	X	X	X	X	9		0.30		0.34	
117601.26	B	X		X			X	X		X	X			X		X	X			9	3.00	0.08	0.98	0.08	0.92
	C	X						X											X	3		0.08		0.08	
106075.97	B	X	X	X						X										4	4.00	0.04	1.60	0.05	1.87
	C						X													1		0.03		0.03	

FIG 7

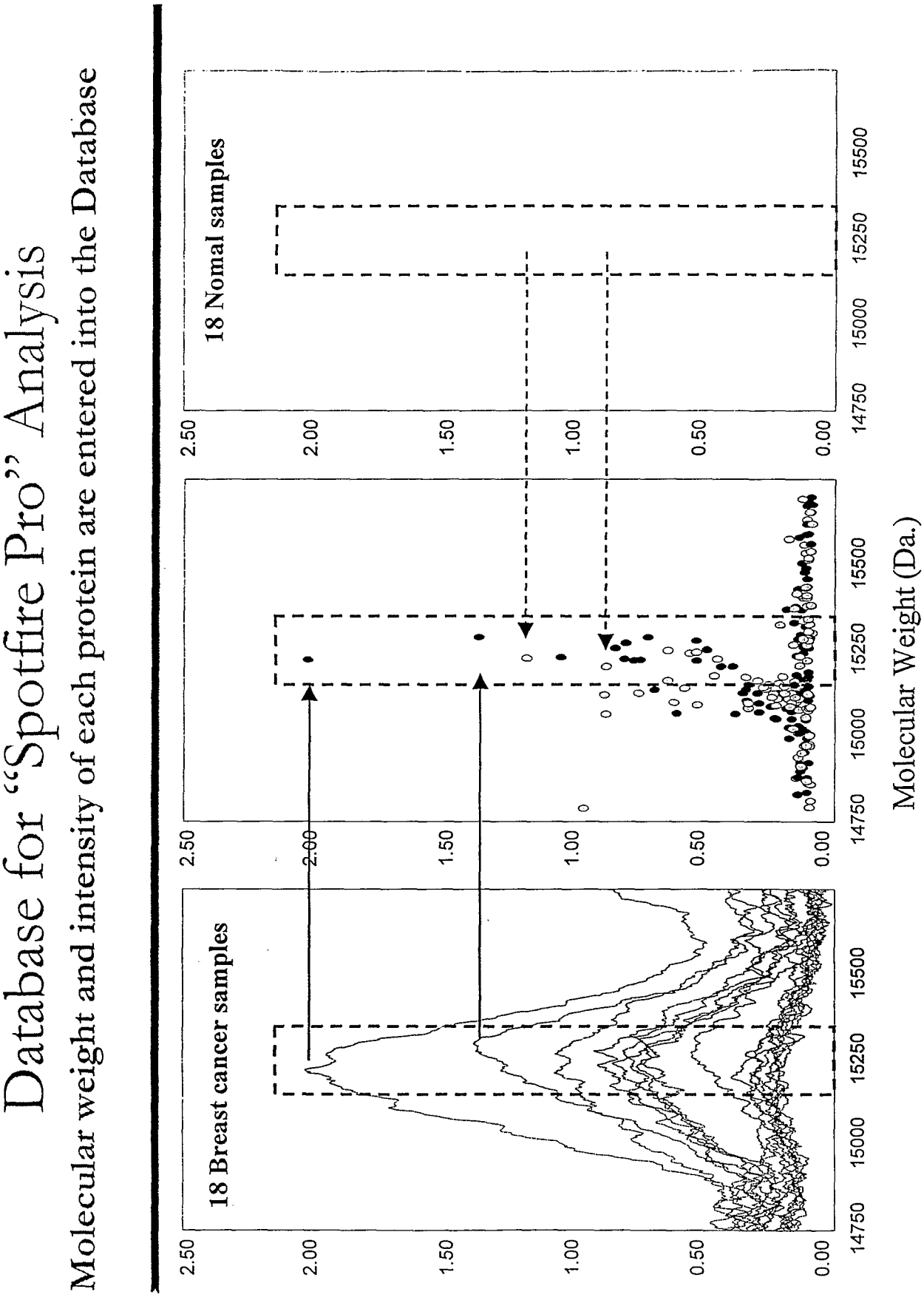

Database for "Spotfire Pro" Analysis
Molecular weight and intensity of each protein are entered into the Database
18 Breast cancer samples
18 Nomal samples
2.50
2.00
1.50
1.00
0.50
0.00
14750
15000
15250
15500
Molecular Weight (Da.)

FIG 8A

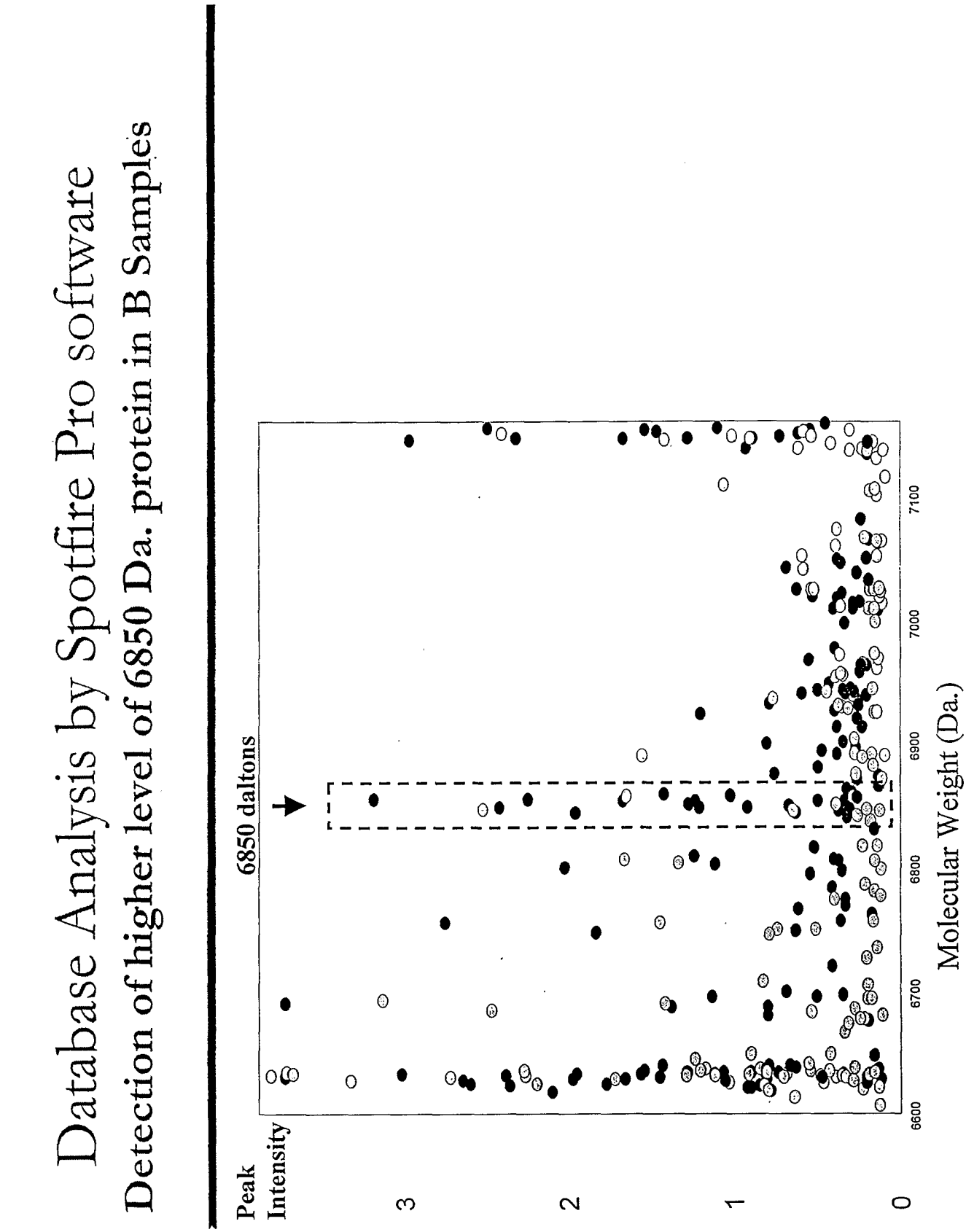

Database Analysis by Spotfire Pro software
Detection of higher level of 6850 Da. protein in B Samples
Peak Intensity
6850 daltons
3
2
1
0
6600
6700
6800
6900
7000
7100
Molecular Weight (Da.)

FIG 8B

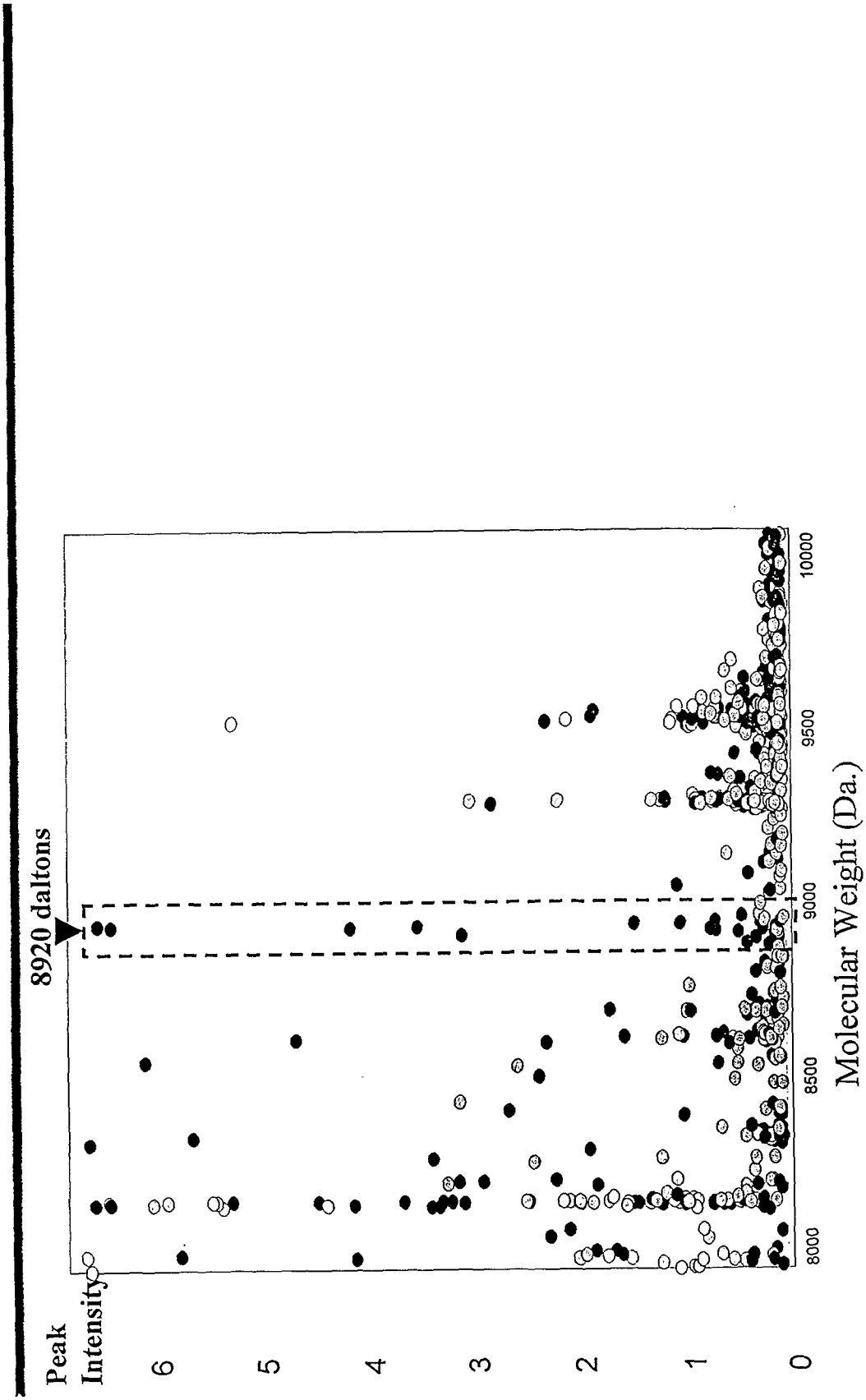

Database Analysis by Spotfire Pro software
Detection of higher level of 8920 Da. protein in B Samples
8920 daltons
Peak Intensity
0
1
2
3
4
5
6
8000
8500
9000
9500
10000
Molecular Weight (Da.)

FIG 8C

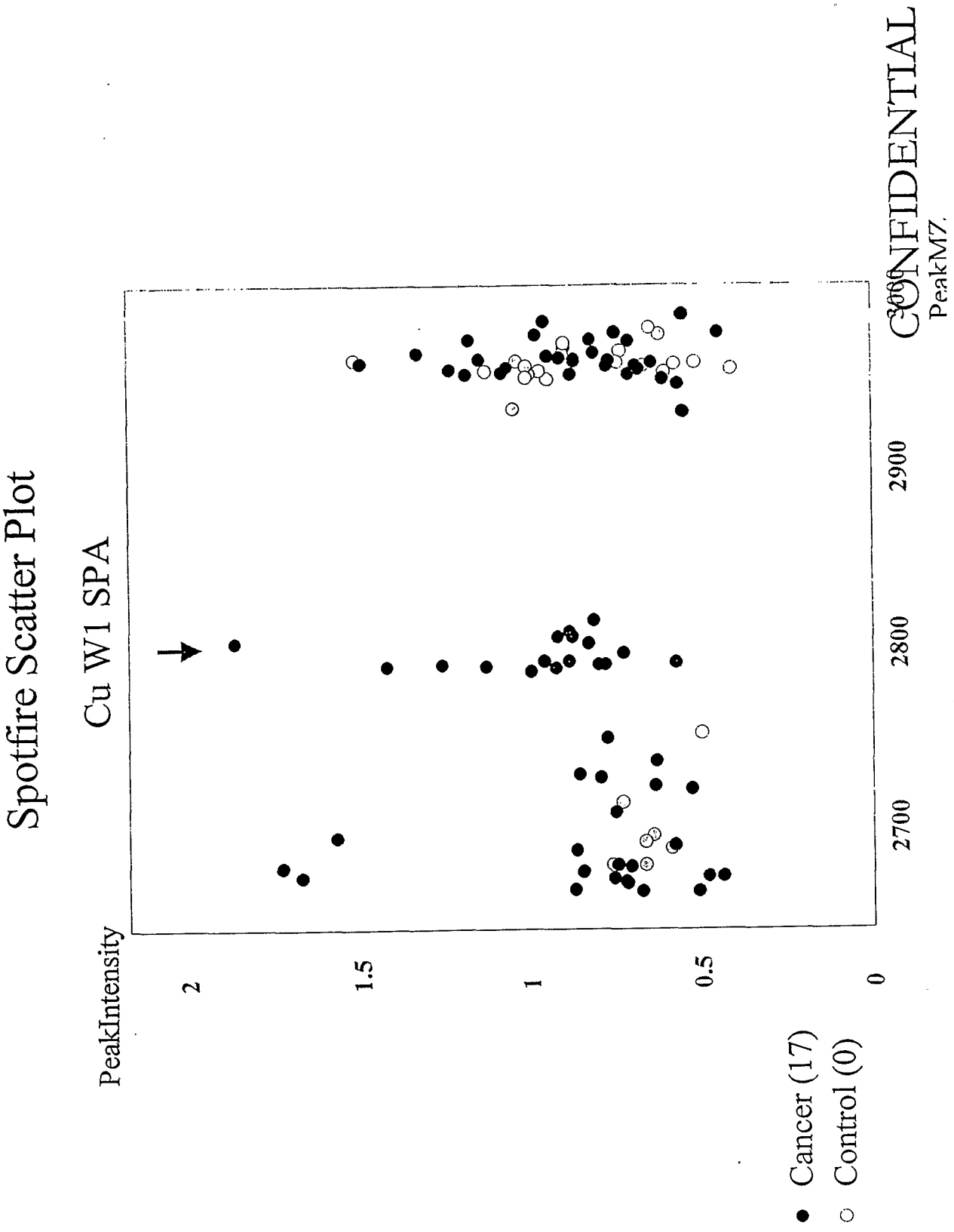

Spotfire Scatter Plot
Cu W1 SPA
PeakIntensity
2
1.5
1
0.5
0
2700
2800
2900
3000
PeakMZ
CONFIDENTIAL
Cancer (17)
Control (0)

FIG 9A

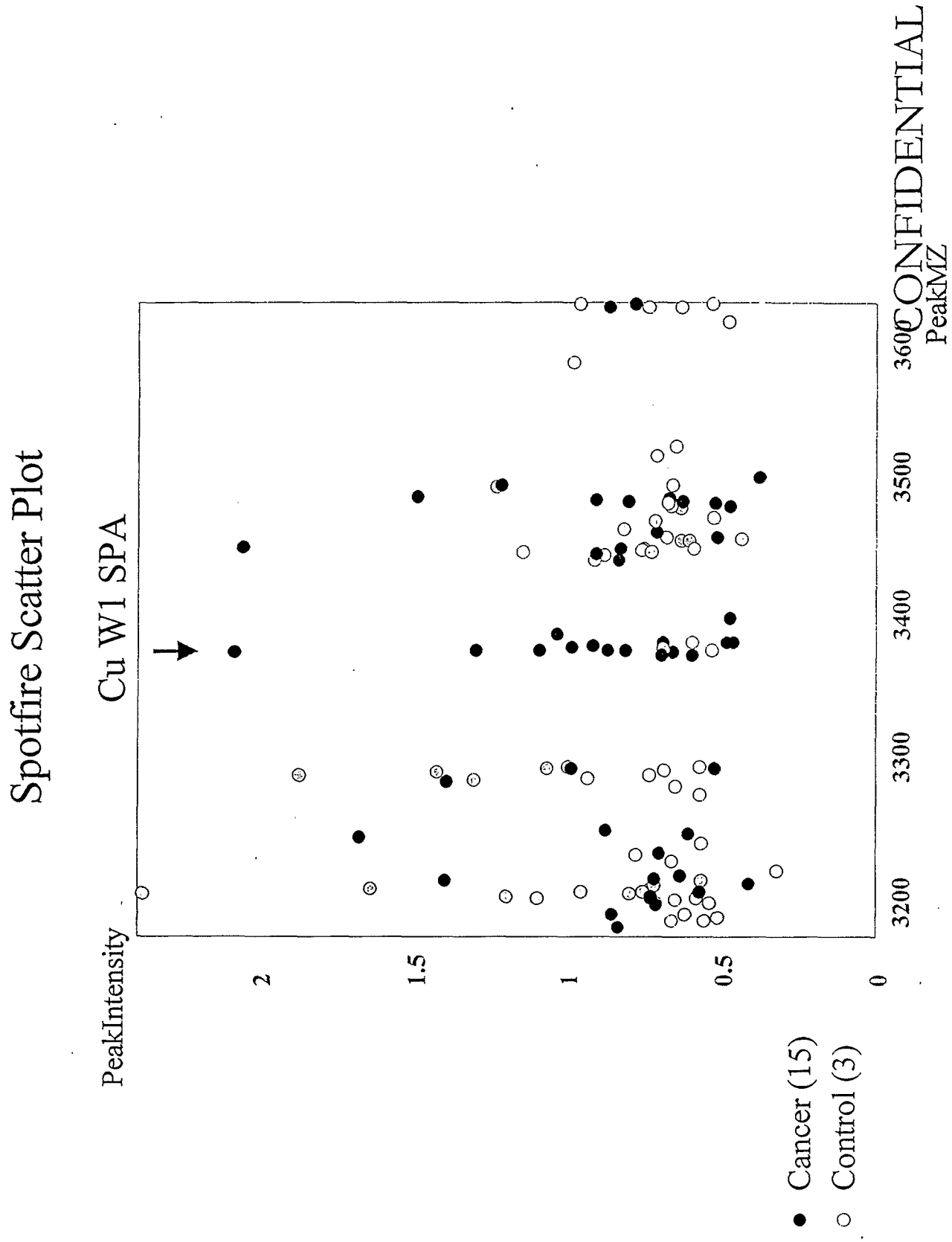

Spotfire Scatter Plot
Cu W1 SPA
PeakIntensity
2
1.5
1
0.5
0
3200
3300
3400
3500
3600
PeakMZ
Cancer (15)
Control (3)
CONFIDENTIAL

FIG 9B

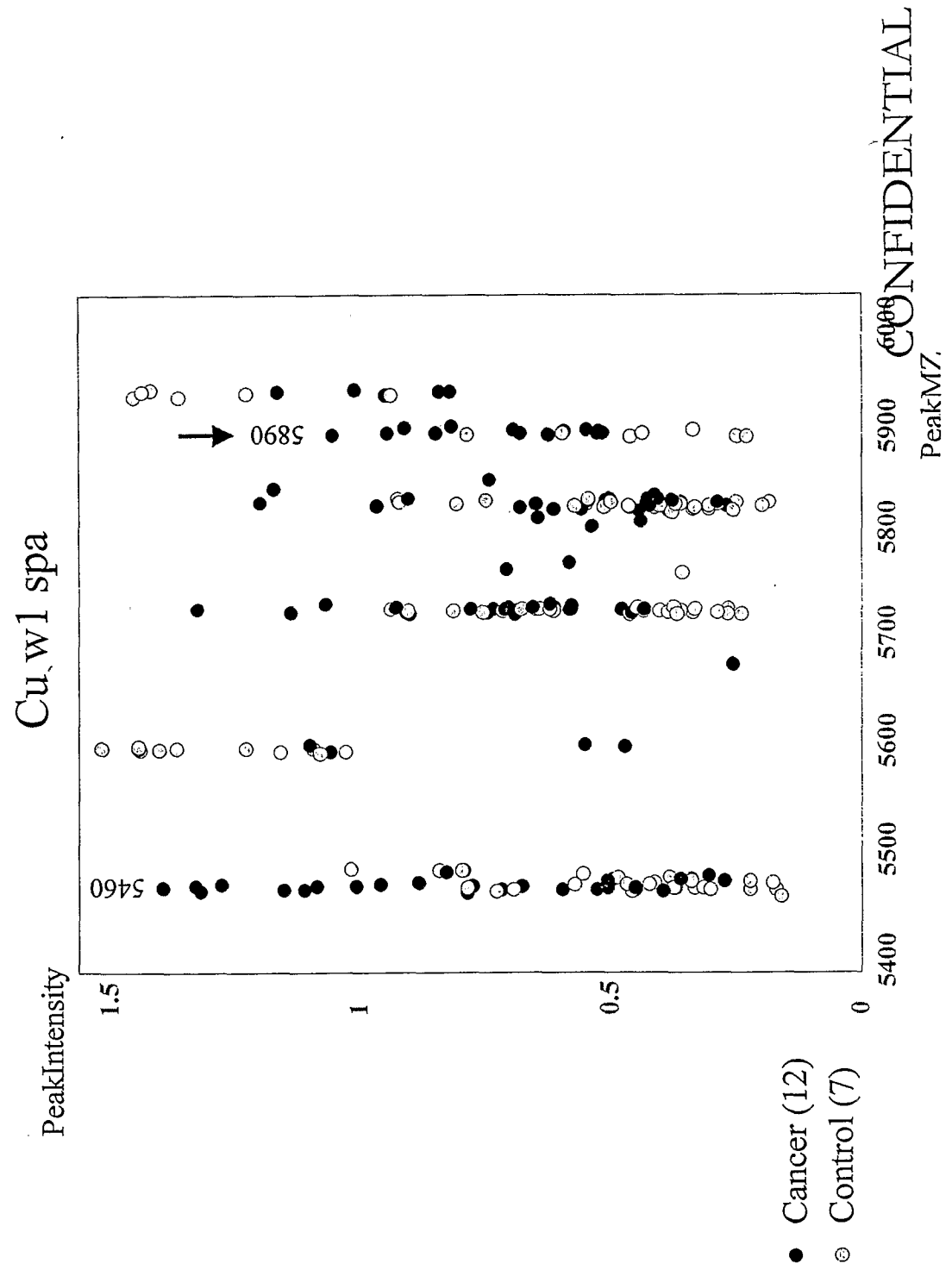

CONFIDENTIAL
Cu_w1 spa
PeakIntensity
1.5
1
0.5
0
5400
5500
5600
5700
5800
5900
6000
PeakMZ
5460
5890
Cancer (12)
Control (7)

FIG 10A

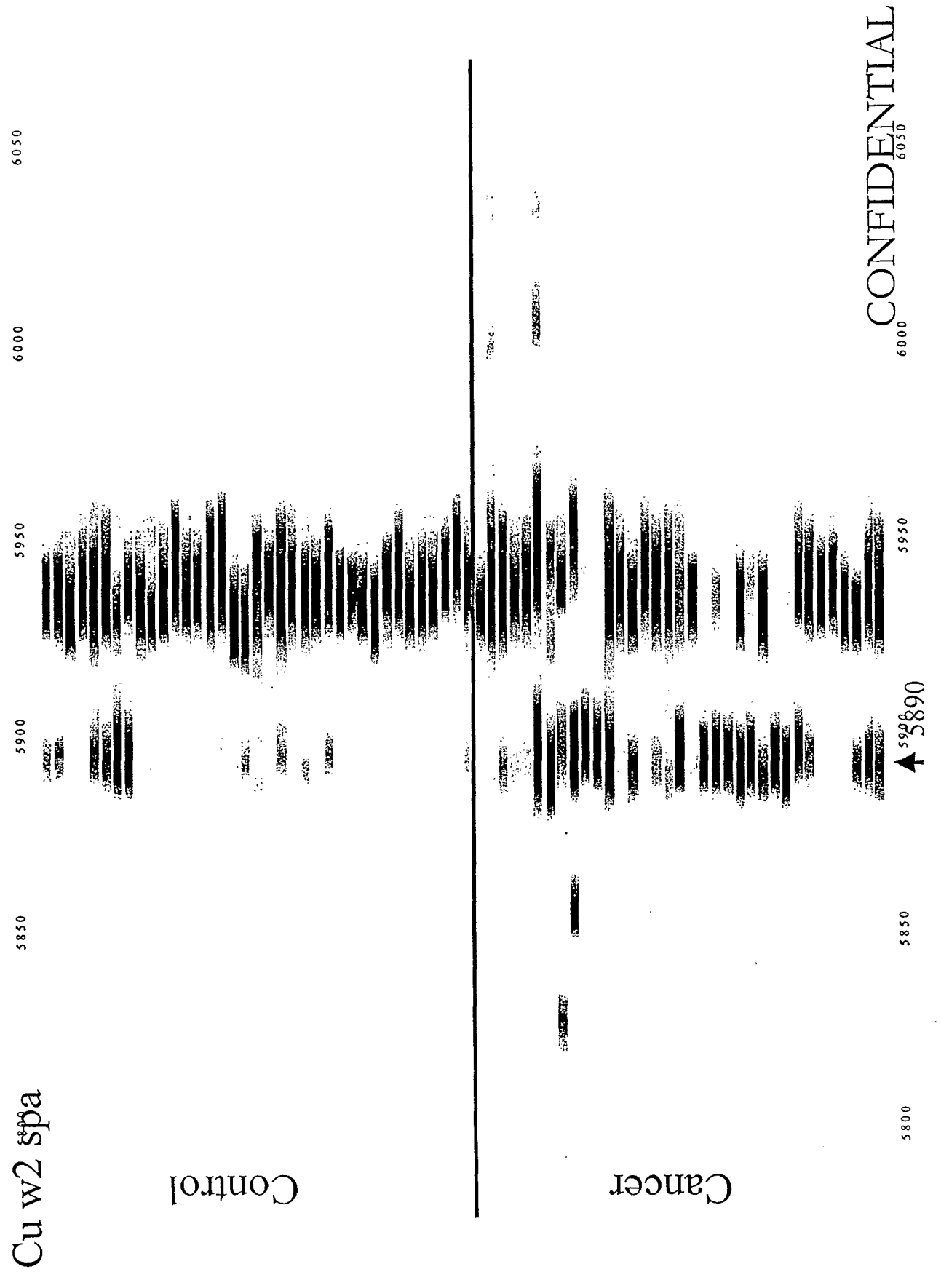

Cu w2 spa
Control
Cancer
CONFIDENTIAL
5800
5850
5900
5950
6000
6050
5890

FIG 10B

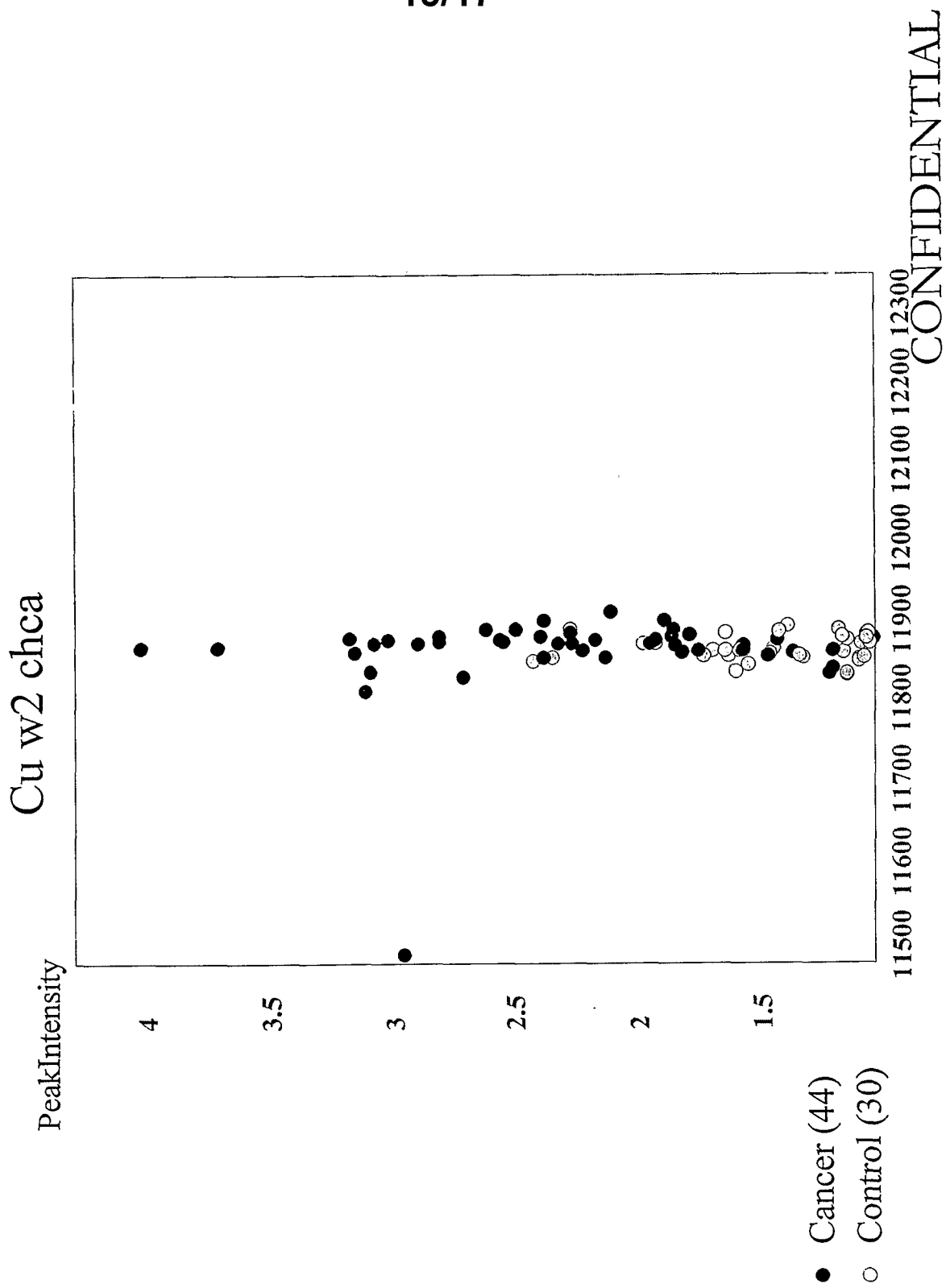

CONFIDENTIAL
Cu w2 chca
PeakIntensity
4
3.5
3
2.5
2
1.5
11500 11600 11700 11800 11900 12000 12100 12200 12300
Cancer (44)
Control (30)

FIG 11A

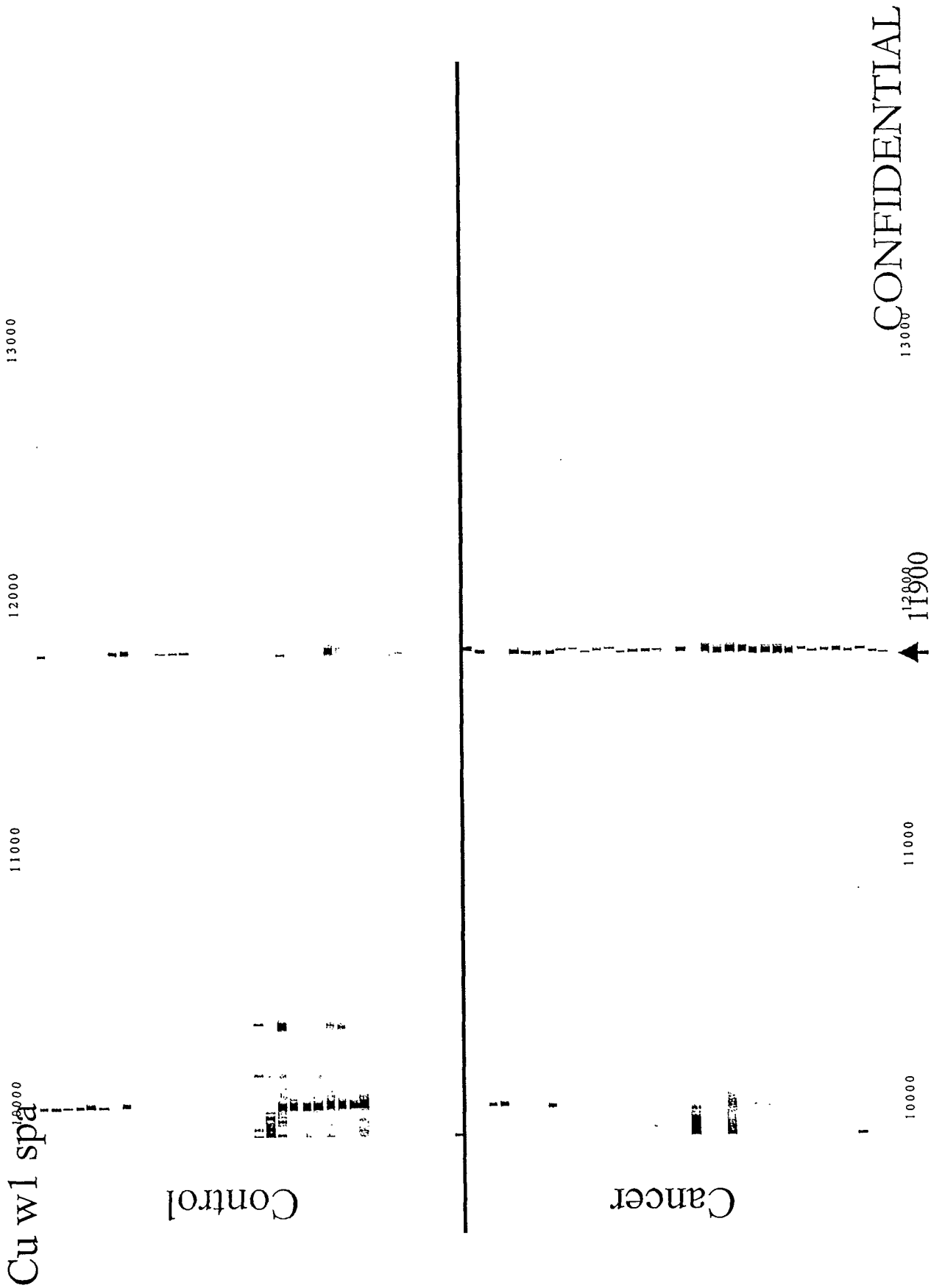

CONFIDENTIAL
13000
12000
11000
10000
11900
Cancer
Control
Cu w1 spa

FIG 11B

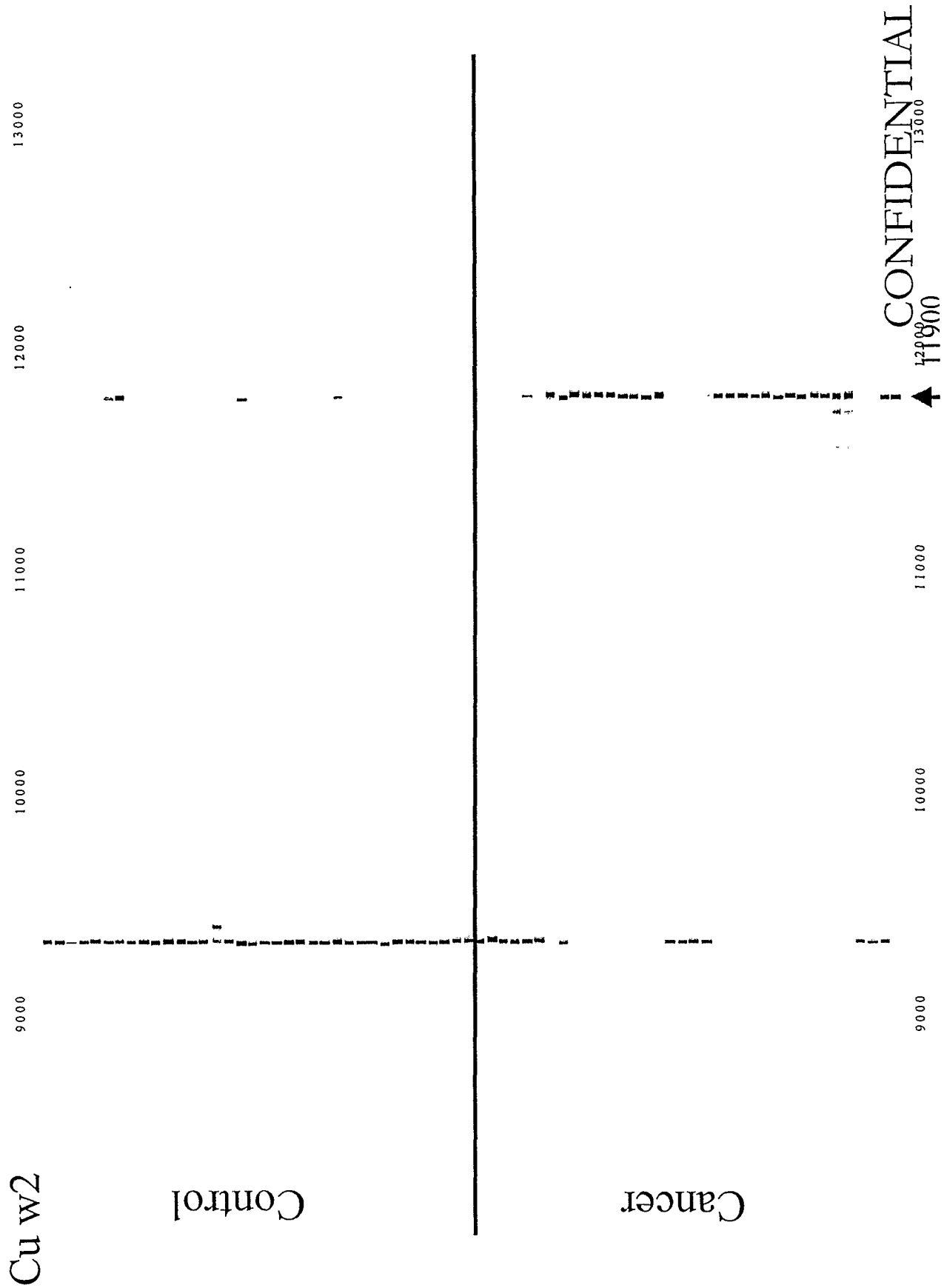

CONFIDENTIAL
11900
13000
12000
11000
10000
9000
Cancer
Control
Cu w2

FIG 11C